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(54) Title: ERYTHROPOIETIN: REMODELING AND GLYCOCONJUGATION OF ERYTHROPOIETIN

(57) Abstract: The invention includes methods and compositions for remodeling a peptide molecule, including the addition or deletion of one or more glycosyl groups to a peptide, and/or the addition of a modifying group to a peptide.

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TITLE OF THE INVENTION

ERYTHROPOIETIN: REMODELING AND GLYCOCONJUGATION OF ERYTHROPOIETIN

BACKGROUND OF THE INVENTION

Most naturally occurring peptides contain carbohydrate moieties attached to the peptide via specific linkages to a select number of amino acids along the length of the primary peptide chain. Thus, many naturally occurring peptides are termed "glycopeptides." The variability of the glycosylation pattern on any given peptide has enormous implications for the function of that peptide. For example, the structure of the N-linked glycans on a peptide can impact various characteristics of the peptide, including the protease susceptibility, intracellular trafficking, secretion, tissue targeting, biological half-life and antigenicity of the peptide in a cell or organism. The alteration of one or more of these characteristics greatly affects the efficacy of a peptide in its natural setting, and also affects the efficacy of the peptide as a therapeutic agent in situations where the peptide has been generated for that purpose.

The carbohydrate structure attached to the peptide chain is known as a "glycan" molecule. The specific glycan structure present on a peptide affects the solubility and aggregation characteristics of the peptide, the folding of the primary peptide chain and therefore its functional or enzymatic activity, the resistance of the peptide to proteolytic attack and the control of proteolysis leading to the conversion of inactive forms of the peptide to active forms. Importantly, terminal sialic acid residues present on the glycan molecule affect the length of the half life of the peptide in the mammalian circulatory system. Peptides whose glycans do not contain terminal sialic acid residues are rapidly removed from the circulation by the liver, an event which negates any potential therapeutic benefit of the peptide.

The glycan structures found in naturally occurring glycopeptides are typically divided into two classes, N-linked and O-linked glycans.

Peptides expressed in eukaryotic cells are typically N-glycosylated on asparagine residues at sites in the peptide primary structure containing the sequence asparagine-X-

serine/threonine where X can be any amino acid except proline and aspartic acid. The earbohydrate portion of such peptides is known as an N-linked glycan. The early events of N-glyeosylation oecur in the endoplasmic reticulum (ER) and are identical in mammals, plants, insects and other higher eukaryotes. First, an oligosaccharide chain comprising fourteen sugar residues is constructed on a lipid carrier molecule. As the nascent peptide is translated and translocated into the ER, the entire oligosaccharide chain is transferred to the amide group of the asparagine residue in a reaction catalyzed by a membrane bound glycosyltransferase enzyme. The N-linked glycan is further processed both in the ER and in the Golgi apparatus. The further processing generally entails removal of some of the sugar residues and addition of other sugar residues in reactions catalyzed by glycosidases and glycosyltransferases specific for the sugar residues removed and added.

Typically, the final structures of the N-linked glycans are dependent upon the organism in which the peptide is produced. For example, in general, peptides produced in bacteria are completely unglycosylated. Peptides expressed in insect cells contain high mannose and paunci-mannose N-linked oligosaccharide chains, among others. Peptides produced in mammalian cell culture are usually glycosylated differently depending, e.g., upon the species and cell culture conditions. Even in the same species and under the same conditions, a certain amount of heterogeneity in the glycosyl chains is sometimes encountered. Further, peptides produced in plant cells comprise glycan structures that differ significantly from those produced in animal cells. The dilemma in the art of the production of recombinant peptides, particularly when the peptides are to be used as therapeutic agents, is to be able to generate peptides that are correctly glycosylated, i.e., to be able to generate a peptide having a glycan structure that resembles, or is identical to that present on the naturally occurring form of the peptide. Most peptides produced by recombinant means comprise glycan structures that are different from the naturally occurring glycans.

A variety of methods have been proposed in the art to customize the glycosylation pattern of a peptide including those described in WO 99/22764, WO 98/58964, WO 99/54342 and U.S. Patent No. 5,047,335, among others. Essentially, many of the enzymes required for the *in vitro* glycosylation of peptides have been cloned and sequenced. In some instances, these enzymes have been used *in vitro* to add specific sugars to an incomplete glycan molecule on a peptide. In other instances, cells have been genetically engineered to express a

combination of enzymes and desired peptides such that addition of a desired sugar moiety to an expressed peptide occurs within the cell.

Peptides may also be modified by addition of O-linked glycans, also called mucin-type glycans because of their prevalence on mucinous glycopeptide. Unlike N-glycans that are linked to asparagine residues and are formed by *en bloc* transfer of oligosaccharide from lipid-bound intermediates, O-glycans are linked primarily to serine and threonine residues and are formed by the stepwise addition of sugars from nucleotide sugars (Tanner *et al.*, *Biochim. Biophys. Acta.* **906**:81-91 (1987); and Hounsell *et al.*, *Glycoconj. J.* **13**:19-26 (1996)). Peptide function can be affected by the structure of the O-linked glycans present thereon. For example, the activity of P-selectin ligand is affected by the O-linked glycan structure present thereon. For a review of O-linked glycan structures, see Schachter and Brockhausen, The Biosynthesis of Branched O-Linked Glycans, 1989, Society for Experimental Biology, pp. 1-26 (Great Britain). Other glycosylation patterns are formed by linking glycosylphosphatidylinositol to the carboxyl-terminal carboxyl group of the protein (Takeda *et al.*, *Trends Biochem. Sci.* **20**:367-371 (1995); and Udenfriend *et al.*, *Ann. Rev. Biochem.* **64**:593-591 (1995).

Although various techniques currently exist to modify the N-linked glycans of peptides, there exists in the art the need for a generally applicable method of producing peptides having a desired, i.e., a customized glycosylation pattern. There is a particular need in the art for the customized *in vitro* glycosylation of peptides, where the resulting peptide can be produced at industrial scale. This and other needs are met by the present invention.

The administration of glycosylated and non-glycosylated peptides for engendering a particular physiological response is well known in the medicinal arts. Among the best known peptides utilized for this purpose is insulin, which is used to treat diabetes. Enzymes have also been used for their therapeutic benefits. A major factor, which has limited the use of therapeutic peptides is the immunogenic nature of most peptides. In a patient, an immunogenic response to an administered peptide can neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of therapeutic peptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the

problems have been investigated. To provide soluble peptide therapeutics, synthetic polymers have been attached to the peptide backbone.

Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to peptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides and prolong the clearance time from the circulation. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) concerns non-immunogenic peptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole peptide and at least 15% of the physiological activity is maintained.

WO 93/15189 (Veronese *et al.*) concerns a method to maintain the activity of polyethylene glycol-modified proteolytic enzymes by linking the proteolytic enzyme to a macromolecularized inhibitor. The conjugates are intended for medical applications.

The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently linked to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1 protease inhibitor with a polymer such as PEG or methoxypoly(ethylene glycol) ("mPEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977) discloses the covalent attachment of mPEG to an amine group of bovine serum albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon-β, interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a peptide. The oxidized sugar is utilized as a loeus for attaching a PEG moiety to the peptide. For example, M'Timkulu (WO 94/05332) discloses the use of a hydrazine- or amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly

oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG. See also, Bona et al. (WO 96/40731), where a PEG is added to an immunoglobulin molecule by enzymatically oxidizing a glycan on the immunoglobulin and then contacting the glycan with an amino-PEG molecule.

In each of the methods described above, poly(ethylene glycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivatization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product.

Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (*e.g.*, sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (*e.g.*, β-mannosidase, β-glucosidase), and endoglycosidases (*e.g.*, Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, *see,* Crout *et al., Curr. Opin. Chem. Biol.* **2**: 98-111 (1998).

Glycosyltransferases modify the oligosaccharide structures on peptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on peptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β-1,4-galactosyltransferase is used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (*see, e.g.,* Wong *et al., J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α-sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (*see, e.g.*, Kevin *et al., Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase

(Ichikawa *et al.*, *J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see,* Koeller *et al.*, *Nature Biotechnology* **18**: 835-841 (2000). *See* also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. However, under appropriate conditions, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. The glycosidase binds a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to yield the hydrolysis product, or by an acceptor, to generate a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the β-mannoside linkage, which is formed by the action of β-mannosidase (Singh *et al., Chem. Commun.* 993-994 (1996)).

In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzyme does not hydrolyze glycosidic linkages, but can still form them. Such a mutant glycosidase is used to prepare oligosaccharides using an α-glycosyl fluoride donor and a glycoside acceptor molecule (Withers et al., U.S. Patent No. 5,716,812).

Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*-β-N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al., Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al., Carbohydr. Res.* **292**: 61-70 (1996)).

In addition to their use in preparing carbohydrates, the enzymes diseussed above are applied to the synthesis of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. et al., J. Am. Chem. Soc. **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc

residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β-1,4-galactosyltransferase, α-2,3-sialyltransferase and α-1,3-fucosyltransferase V. However, while each enzymatically catalyzed step proceeded in excellent yield, such procedures have not been adapted for the generation of glycopeptides on an industrial scale.

Methods combining both chemical and enzymatic synthetic elements are also known in the art. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglycosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin peptide. The saccharide portion was added to the peptide by treating it with an endo-β-N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and peptides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested in the prior art.

Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al., J. Biol. Chem.* **271**: 27213 (1996)).

Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing modifying group. For example, Casares *et al.* (*Nature Biotech.* **19**: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

Glycosyl residues have also been modified to contain ketone groups. For example, Mahal and co-workers (*Science* **276**: 1125 (1997)) have prepared N-levulinoyl mannosamine ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. *See,* also Saxon *et al., Science* **287**: 2007 (2000); Hang *et al., J. Am. Chem. Soc.* **123**: 1242 (2001); Yarema *et al., J. Biol. Chem.* **273**: 31168 (1998); and Charter *et al., Glycobiology* **10**: 1049 (2000).

The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Further, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

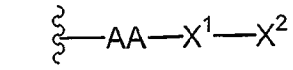
Despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for an industrially practical method for the modification of glycosylated and non-glycosylated peptides with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules and the like. Of particular interest are methods in which the modified peptide has improved properties, which enhance its use as a therapeutic or diagnostic agent. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The invention includes a multitude of methods of remodeling a peptide to have a speeific glycan structure attached thereto. Although specific glycan structures are described herein, the invention should not be construed to be limited to any one particular structure. In addition, although specific peptides are described herein, the invention should not be limited by the nature of the peptide described, but rather should encompass any and all suitable peptides and variations thereof.

The description which follows discloses the preferred embodiments of the invention and provides a written description of the claims appended hereto. The invention encompasses any and all variations of these embodiments that are or become apparent following a reading of the present specification.

The invention includes a cell-free, in vitro method of remodeling an erythropoietin (EPO) peptide, the peptide having the formula:



wherein

AA is a terminal or internal amino acid residue of the peptide;

X^1-X^2 is a saccharide covalently linked to the AA, wherein

X^1 is a first glycosyl residue; and

X^2 is a second glycosyl residue covalently linked to X^1, wherein X^1 and X^2 are selected from monosaccharyl and oligosaccharyl residues;

the method comprising:

(a) removing X^2 or a saccharyl subunit thereof from the peptide, thereby forming a truncated glycan; and

(b) contacting the truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, thereby remodeling the EPO peptide.

The method further comprises:

(c) removing X^1, thereby exposing the AA; and

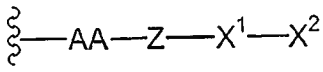
(d) contacting the AA with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the AA, thereby remodeling the EPO peptide.

The method additionally comprises:

(e) prior to step (b), removing a group added to the saccharide during post-translational modification.

In one aspect, the group is a member selected from phosphate, sulfate, carboxylate and esters thereof.

In one aspect of the method, the peptide has the formula:



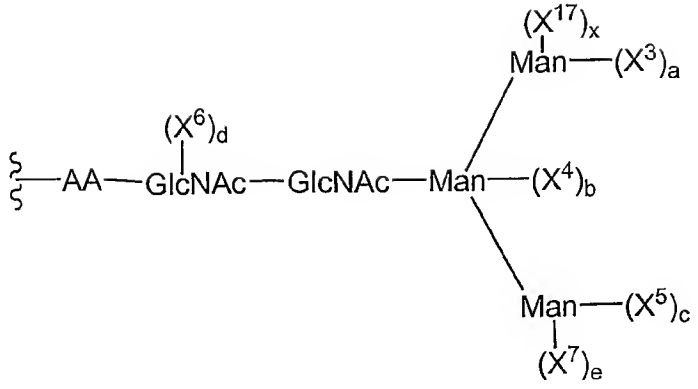
wherein

Z is a member selected from O, S, NH and a crosslinker.

In one embodiment, at least one of the glycosyl donors comprises a modifying group.

In this and in other embodiments, the modifying group is a member selected from the group consisting of a polymer, a therapeutic moiety, a detectable label, a reactive linker group, a targeting moiety, and a peptide. Preferably, the modifying group is a water soluble polymer, preferably, poly(ethylene glycol), wherein preferably, the poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

There is also provided a cell-free in vitro method of remodeling an EPO peptide, the peptide having the formula:



wherein

X^3, X^4, X^5, X^6, X^7 , and X^{17} are independently selected monosaccharyl or oligosaccharyl residues; and

a, b, c, d, e and x are independently selected from the integers 0, 1 and 2, with the proviso that at least one member selected from a, b, c, d, and e and x are 1 or 2; the method comprising:

(a) removing at least one of X^3, X^4, X^5, X^6, X^7, or X^{17}, a saccharyl subunit thereof from the peptide, thereby forming a truncated glycan; and

(b) contacting the truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, thereby remodeling the EPO peptide.

In one embodiment, the removing of step (a) produces a truncated glycan in which a, b, c, e and x are each 0.

In another embodiment, X^3, X^5, and X^7, are selected from the group consisting of $(mannose)_z$ and $(mannose)_z$-$(X^8)_y$

wherein

X^8 is a glycosyl moiety selected from mono- and oligo-saccharides;

y is an integer selected from 0 and 1; and

z is an integer between 1 and 20, wherein

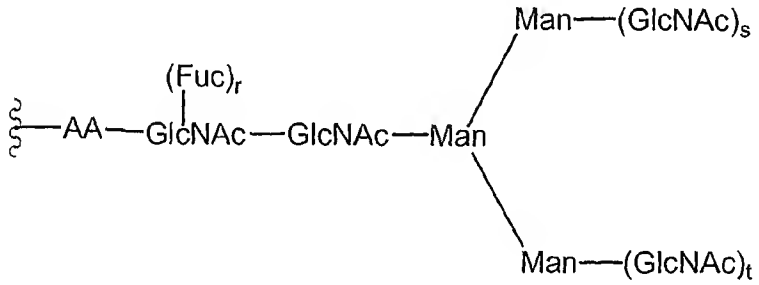
when z is 3 or greater, $(mannose)_z$ is selected from linear and branched structures.

In a further embodiment, X^4 is selected from the group consisting of GlcNAc and xylose.

In yet another embodiment, X^3, X^5, and X^7 are $(mannose)_u$, wherein u is selected from the integers between 1 and 20, and when u is 3 or greater, $(mannose)_u$ is selected from linear and branched structures.

In one aspect, at least one of the glycosyl donors comprises a modifying group.

There is further provided a cell-free in vitro method of remodeling an EPO peptide comprising a glycan having the formula:



wherein

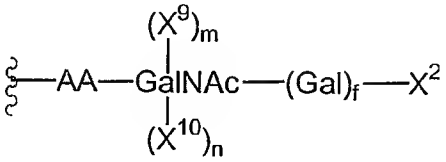
r, s, and t are integers independently selected from 0 and 1,

the method comprising:

(a) contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the glycan, thereby remodeling the EPO peptide.

In one aspeet, at least one of the glycosyl donors comprises a modifying group.

In another aspect, the peptide has the formula:

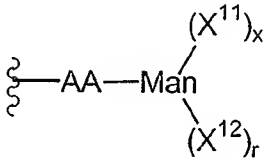


wherein

X^9 and X^{10} are independently selected monosaccharyl or oligosaccharyl residues; and

m, n and f are integers selected from 0 and 1.

Further, the peptide has the formula:



wherein

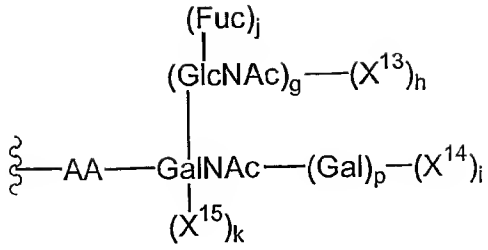
X^{11} and X^{12} are independently selected glycosyl moieties; and

r and x are integers independently selected from 0 and 1.

In another aspect, X^{11} and X^{12} are $(mannose)_q$, wherein

q is selected from the integers between 1 and 20, and when q is three or greater, $(mannose)_q$ is selected from linear and branched structures.

In addition, the peptide has the formula:



wherein

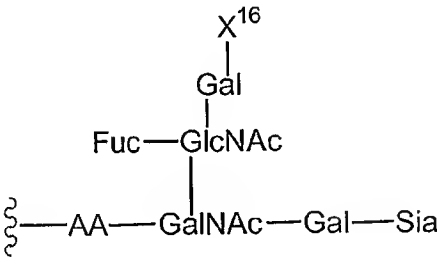
X^{13}, X^{14}, and X^{15} are independently selected glycosyl residues; and

g, h, i, j, k, and p are independently selected from the integers 0 and 1, with the proviso that at least one of g, h, i, j, k and p is 1.

The method also includes wherein

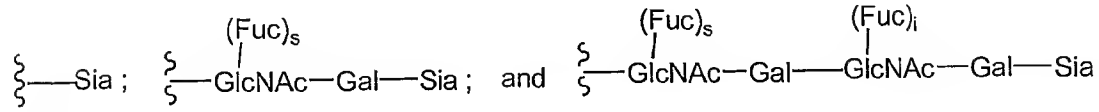
X^{14} and X^{15} are members independently selected from GlcNAc and Sia; and i and k are independently selected from the integers 0 and 1, with the proviso that at least one of i and k is 1 and if k is 1, g, h and j are 0.

In an additional aspect, the peptide has the formula:



wherein

X^{16} is a member selected from:

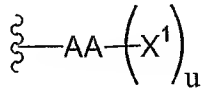


wherein

s and i are integers independently selected from 0 and 1.

In one embodiment, the removing utilizes a glycosidase.

Also included is a cell-free, in vitro method of remodeling an EPO peptide having the formula:



wherein

AA is a terminal or internal amino acid residue of the peptide;

X^1 is a glycosyl residue covalently linked to the AA, selected from monosaccharyl and oligosaccharyl residues; and

u is an integer selected from 0 and 1,

the method comprising:

contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the truncated glycan, wherein the glycosyl donor comprises a modifying group, thereby remodeling the EPO peptide.

The invention further provides a covalent conjugate between an EPO peptide and a modifying group that alters a property of the peptide, wherein the modifying group is covalently attached to the peptide at a preselected glycosyl or amino acid residue of the peptide via an intact glycosyl linking group.

In one aspect, the modifying group is a member selected from the group consisting of a polymer, a therapeutic moiety, a detectable label, a reactive linker group, a targeting moiety, and a peptide. In another aspect, the modifying group and an intact glycosyl linking group precursor are linked as a covalently attached unit to the peptide via the action of an enzyme, the enzyme converting the precursor to the intact glycosyl linking group, thereby forming the conjugate. In a further aspect, the covalent conjugate eomprises a

first modifying group covalently linked to a first residue of the peptide via a first intaet glycosyl linking group, and a second glycosyl linking group linked to a second residue of the peptide via a second intact glycosyl linking group. In one embodiment, the first residue and the second residue are structurally identical. In another embodiment, the first residue and the second residue have different structures. In an additional embodiment, the first residue and the second residue are glycosyl residues. Further, the first residue and the second residue are amino acid residues. Also, the peptide may be remodeled prior to forming the conjugate. In another aspect, the remodeled peptide is remodeled to introduce an acceptor moiety for the intact glycosyl linking group. In yet a further aspect, the modifying group is a water-soluble polymer. In one aspect, the intact glycosyl linking unit is a member selected from the group consisting of a sialic acid residue, a Gal residue, a GlcNAc residue, and a GalNAc residue.

There is a also provided a method of forming a covalent conjugate between a polymer and a glycosylated or non-glycosylated peptide, wherein the polymer is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, the method comprising:

contacting the peptide with a mixture comprising a nucleotide sugar covalently linked to the polymer and a glycosyltransferase for which the nucleotide sugar is a substrate under conditions sufficient to form the conjugate, wherein the peptide is EPO.

In one aspect, the polymer is a water-soluble polymer. In another aspect, the glycosyl linking group is covalently attached to a glycosyl residue covalently attached to the peptide. In yet a further aspect, the glycosyl linking group is covalently attached to an amino acid residue of the peptide. In an additional aspect, the polymer comprises a member selected from the group consisting of a polyalkylene oxide and a polypeptide. An in another aspect, the polyalkylene oxide is poly(ethylene glycol).

In yet a further aspect, the glycosyltransferase is seleeted from the group consisting of sialyltransferase, galactosyltransferase, glucosyltransferase, GalNAc

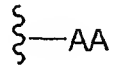
transferase, and a GlcNAc transferase. Additionally, the glycosyltransferase is recombinantly produced and may be either a recombinant prokaryotic or eukaryotic enzyme.

In one embodiment, the nucleotide sugar is selected from the group consisting of UDP-glycoside, CMP-glycoside, and GDP-glycoside. Further, the nucleotide sugar is selected from the group consisting of UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, UDP-N-acetylgalactosamine, UDP-N-acctylglucosaminc, GDP-mannose, GDP-fucose, CMP-sialic acid, CMP-NeuAc. In addition, the glycosylated peptide is partially deglycosylated prior to the contacting. Further, the intact glycosyl linking group is a sialic acid residue. In addition, the method is performed in a cell-free environment. Also, the covalent conjugate is isolated, preferably by membrane filtration.

The invention further provides a composition for forming a conjugate between a peptide and a modified sugar, the composition comprising: an admixture of a modified sugar, a glycosyltransferase, and a peptide acceptor substrate, wherein the modified sugar has covalently attached thereto a member selected from a polymer, a therapeutic moiety and a biomolecule, wherein the peptide is EPO.

In addition, there is provided an EPO peptide remodeled by the methods of the invention and pharmaceutical compositions comprising such EPO peptides.

Also provided is a cell-free, in vitro method of remodeling a peptide having the formula:



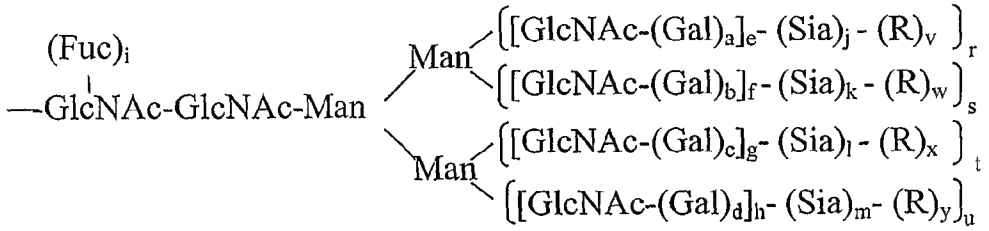
wherein

AA is a terminal or internal amino acid residue of the peptide,

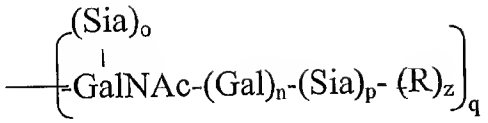
the method comprising:

contacting the peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer the at least one glycosyl donor to the amino acid residue, wherein the glycosyl donor comprises a modifying group, thereby remodeling the peptide, wherein the peptide is EPO.

In addition, there is provided a method for forming a conjugate between an EPO peptide and a modifying group, wherein the modifying group is covalently attached to the EPO peptide through an intact glycosyl linking group, the EPO peptide comprising a glycosyl residue having a formula which is a member selected from:



; and



wherein

a, b, c, d, i, n, o, p, q, r, s, t, and u are members independently selected from 0 and 1;

e, f, g, and h are members independently selected from the integers between 0 and 4;

j, k, l, and m are members independently selected from the integers between 0 and 20;

v, w, x, y, and z are 0; and

R is a modifying group, a mannose or an oligomannose;

the method comprising:

(a) contacting the EPO peptide with a glycosyltransferase and a modified glycosyl donor, comprising a glycosyl moiety which is a substrate for the glycosyltransferase covalently linked to the modifying group, under conditions appropriate for the formation of the intact glycosyl linking group.

The method further comprises:

(b) prior to step (a), contacting the EPO peptide with a sialidase under conditions appropriate to remove sialic acid from the EPO peptide.

The method additionally comprises:

(c) contacting the product of step (a) with a sialyltransferase and a sialic acid donor under conditions appropriate to transfer sialic acid to the product.

The method further comprises:

(d) prior to step (a), contacting the EPO peptide with a galactosidase operating synthetically under conditions appropriate to add a galactose to the EPO peptide.

In addition, the method comprises:

(e) prior to step (a), contacting the EPO peptide with a galactosyl transferase and a galactose donor under conditions appropriate to transfer the galactose to the EPO peptide.

The method also comprises:

(f) contacting the product from step (e) with ST3Gal3 and a sialic acid donor under conditions appropriate to transfer sialic acid to the product.

The method also comprises:

(g) contacting the product from step (a) with a moiety that reacts with the modifying group, thereby forming a conjugate between the intact glycosyl linking group and the moiety.

In addition, the method comprises:

(h) prior to step (a), contacting the EPO peptide with N-acetylglucosamine transferase and a GlcNAc donor under conditions appropriate to transfer GlcNAc to the EPO peptide.

In one asepct, the modifying group is a member selected from a polymer, a toxin, a radioisotope, a therapeutic moiety and a glycoconjugate.

In another aspect,

a, b, c, d, e, f, g, n, and q are 1;

h is a member selected from the integers between 1 and 3;

i, j, k, l, m, o, p, r, s, t, and u are members independently selected from 0 and 1; and

v, w, x, y and z are 0.

In yet another aspect,

a, b, c, d, f, h, j, k, l, m, q, s, u, v, w, x, y, and z are 0; and

e, g, i, r, and t are members independently selected from 0 and 1.

In an additional aspect,

a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, and u are members independently selected from 0 and 1; and

v, w, x, y, and z are 0.

In a further aspect,

a, b, c, d, e, f, g, n, and q are 1;

h is a member selected from the integers between 1 and 3;

i, j, k, l, m, o, p, r, s, t, and u are members independently selected from 0 and 1; and

v, w, x, y and z are 0.

In another aspect,

a, b, c, d, f, h, j, k, l, m, o, p, s, u, v, w, x, y, and z are 0; and

e, g, i, n, q, r, and t are independently selected from 0 and 1.

Additionally,

a, b, c, d, f, h, j, k, l, m, n, o, p, s, u, v, w, x, y, and z are 0; and

e, g, i, q, r, and t are members independently selected from 0 and 1.

Further

q is 1;

a, b, c, d, e, f, g, h, i, n, r, s, t, and u are members independently selected from 0 and 1; and

j, k, l, m, o, p, v, w, x, y, and z are 0.

There is also provided an EPO peptide conjugate formed by the methods of the invention.

Further provided is an EPO peptide comprising one or more glycans, having a glycoconjugate molecule covalently attached to the peptide. In one aspect, the one or more glycans is a monoantennary glycan. In another aspect, the one or more glycans is a biantennary glycan. In a further aspect, the one or more glycans is a triantennary glycan. In

yet another aspect, the one or more glycans is at least a triantennary glycan. In a further aspect, the one or more glycans comprises at least two glycans comprising a mixture of mono or multiantennary glycans. In yet another aspect, the one or more glycans is selected from an N-linked glycan and an O-linked glycan. In a further aspect, the one or more glycans is at least two glycans selected from an N-linked and an O-linked glycan. Additionally, the peptide is expressed in a cell selected from the group consisting of a prokaryotic cell and a eukaryotic cell, and the eukaryotic cell is selected from the group consisting of a mammalian cell, an insect cell and a yeast cell.

Also provided in the invention is a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase. In one aspect, the glycoPEGylated EPO peptide comprises at least one mono-antennary glycan. In another aspect, all of the glycans are N-linked and are mono-antennary. In a further aspect, all of the glycans are N-linked and at least one of the glycans comprise the poly(ethylene glycol). In an additional aspect, more than one of the glycans comprises the poly(ethylene glycol). In a further aspect, all of the glycans are N-linked and all of the glycans comprise the poly(ethylene glycol). Additionally, the glycoPEGylated peptide comprises at least three mono-antennary glycans having the poly(ethylene glycol) covalently attached thereto.

There is further provided a glycoPEGylated EPO peptide, wherein the EPO peptide comprises three or more glycans. In one aspect, at least one of the glycans comprises the poly(ethylene glycol) covalently attached thercto. In another aspect, more than one of the glycans comprises the poly(ethylene glycol) covalently attached thereto. In a further aspect, all of the glycans comprise the poly(ethylene glycol) covalently attached thereto. Additionally, the poly(ethylene glycol) is linked to at least one sugar moiety selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA). Additionally, the sialic acid is N-acetylneuraminic acid. Further, the EPO peptide does not comprise an O-linked glycan. In addition, the EPO peptide comprises at least one O-linked glycan. Further, the O-linked peptide comprises the poly(ethylene

glycol) covanently attached thereto. In addition, the EPO peptide is recombinantly expressed in a cell. Further, the cell is selected from the group consisting of an insect cell, a yeast cell and a mammalian cell. Preferably, the the mammalian cell is a CHO cell. In one aspect, the poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa and 40 kDa. In another aspect, the EPO peptide is selected from the group consisting of a naturally occurring EPO peptide and a mutated EPO peptide. In another aspect, mutated EPO peptide comprises the amino acid sequence of SEQ ID NO:73 having at least one mutation selected from the group consisting of Arg^{139} to Ala^{139}, Arg^{143} to Ala^{143} and Lys^{154} to Ala^{154}.

There is also provided a method of making a glycoPEGylated EPO peptide, the method comprising the steps of:

(a) contacting an EPO peptide with a mixture comprising a nucleotide sugar covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions sufficient to transfer the poly(ethylene glycol) to the EPO peptide.

In one embodiment, the sugar of the nucleotide sugar is selected from the group consisting of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal) and a sialic acid (SA). In another embodiment, the sialic acid is N-acetylneuraminic acid (NAN). In a further embodiment, the poly(ethylene glycol) has a molecular weight selected from the group consisting of about 1 kDa, 2 kDa, 5 kDa, 10 kDa, 20 kDa, 30 kDa and 40 kDa. In an additional embodiment, the EPO peptide is recombinantly expressed in a cell, where the cell may be selected from the group consisting of an insect cell, a yeast cell and a mammalian cell. In one aspect, the EPO peptide is selected from the group consisting of a naturally occurring EPO peptide and a mutated EPO peptide. In another aspect, the mature EPO has the sequence of SEQ ID NO:73. In a further aspect, the mutated EPO peptide comprises the amino acid sequence of SEQ ID NO: 73 having at least one mutation selected from the group consisting of Arg^{139} to Ala^{139}, Arg^{143} to Ala^{143} and Lys^{154} to Ala^{154}.

In another aspect of the method of the invention, before step (a): the method comprises:

(b) contacting the EPO peptide with a mixture comprising a nucleotide-N-acetylglucosamine (GlcNAc) molecule and an N-acetylglucosamine transferase (GnT) for which the nucleotide-GlcNAc is a substrate under conditions sufficient to form a bond between the GlcNAc and the EPO, wherein the GnT is selected from the group consisting of GnT I, GnT II, GnT III, GnT IV, GnT V and GnT VI.

In one aspect, the mixture comprises one GnT selected from the group consisting of GnT I, GnT II, GnT IV, GnT V and GnT VI. In another aspect, the glycoPEGylated EPO peptide comprises at least one mono-antennary glycan. In a further aspect, the sugar of the nucleotide sugar is galactose and the glycosyltransferase is galactosyl transferase I (GalT I).

In an additional aspect of the method, before step (a) but after step (b):

(c) contacting the EPO peptide with a mixture comprising a nucleotide galactose (Gal) and galactosyl transferase I (GalT I) under conditions sufficient to transfer galactose to the EPO peptide.

In one embodiment, in step (a), the sugar of the nucleotide sugar is sialic acid and the glycosyltransferase is a sialyltransferase. In another embodiment, the sialic acid is N-acetylneuraminic acid (NAN). In a further embodiment, the sialyltransferase is selected from the group consisting of α(2,3)sialyltransferase, α(2,6)sialyltransferase and (2,8)sialyltransferase.

There is also provided a glycoPEGylated EPO, the EPO comprising the sequence of SEQ ID NO:73. In one aspect, the EPO comprises the sequence of SEQ ID NO:73 and further comprising a mutation in the sequence.

There is further provided in the invention a method of making a glycoPEGylated EPO peptide, the method comprising the steps of:

(a) contacting an EPO peptide with a mixture comprising a nucleotide sugar covalently linked to poly(ethylene glycol) and a glycosyltransferase under conditions

sufficient to transfer the poly(ethylene glycol) to the EPO peptide, wherein the glycosyltransferase is a fucosyltransferase.

In one aspect, the fucosyltransferase is selected from the group consisting of fucosyltransferase I, fucosyltransferase III, fucosyltransferase IV, fucosyltransferase V, fucosyltransferase VI and fucosyltransferase VII, and in another, EPO is expressed in a CHO cell.

There is further provided a method of treating a mammal having anemia, the method comprising administering to the mammal an EPO peptide having one or more glycans having a glycoconjugate molecule attached to the peptide, wherein the EPO is administered in an amount effective to increase the hematocrit level in the mammal. In this and in other embodiments, the mammal is a human.

Also provided is a method of providing erythropoietin therapy to a mammal, the method comprising administering an effective amount of a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount effective to increase the hematocrit level in the mammal.

In addition, there is provided a method of treating a mammal having anemia, the method comprising administering to the mammal a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount effective to increase the hematocrit level in the mammal. In one aspect, the anemia is associated with chemotherapy.

Further provided is a method of treating a kidney dialysis patient, the method comprising administering to the patient a glycoPEGylated EPO peptide comprising an EPO peptide and at least one glycan and at least one poly(ethylene glycol) molecule covalently attached to the glycan, wherein the poly(ethylene glycol) molecule is added to the EPO using a glycosyltransferase, wherein the EPO is administered in an amount effective to increase the hematocrit level in the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 is a scheme depicting a trimannosyl core glycan (left side) and the enzymatic process for the generation of a glycan having a bisecting GlcNAc (right side).

Figure 2 is a scheme depicting an clemental trimannosyl core structure and complex chains in various degrees of completion. The *in vitro* enzymatic generation of an elemental trimannosyl core structure from a complex carbohydrate glycan structure which does not contain a bisecting GlcNAc residue is shown, as is the generation of a glycan structure therefrom which contains a bisecting GlcNAc. Symbols: squares: GlcNAc; light circles: Man; dark circles: Gal; triangles: NeuAc.

Figure 3 is a scheme for the enzymatic generation of a sialylated glycan structure (right side) beginning with a glycan having a trimannosyl core and a bisecting GlcNAc (left side).

Figure 4 is a scheme of a typical high mannose containing glycan structure (left side) and the enzymatic process for reduction of this structure to an elemental trimannosyl core structure. In this scheme, X is mannose as a monosaccharide, an oligosaccharide or a polysaccharide.

Figure 5 is a diagram of a fucose and xylose containing N-linked glycan structure typically produced in plant cells.

Figure 6 is a diagram of a fucose containing N-linked glycan structure typically produced in insect cells. Note that the glycan may have no core fucose, it amy have a single core fucose with either linkage, or it may have a single core fucose having a preponderance of one linkage.

Figure 7 is a scheme depicting a variety of pathways for the trimming of a high mannose structure and the synthesis of complex sugar chains therefrom. Symbols: squares: GlcNAc; circles: Man; diamonds: fucose; pentagon: xylose.

Figure 8 is a scheme depicting *in vitro* strategies for the synthesis of complex structures from an elemental trimannosyl core structure. Symbols: Squares: GlcNAc; light circles: Man; dark circles: Gal; dark triangles: NeuAc; GnT: N-acetyl glucosaminyltransferase; GalT: galactosyltransferase; ST: sialyltransferase.

Figure 9 is a scheme depicting two *in vitro* strategies for the synthesis of monoantennary glycans, and the optional glycoPEGylation of the same. Dark squares: GlcNAc; dark circles: Man; light circles: Gal; dark triangles: sialic acid.

Figure 10 is a scheme depicting two *in vitro* strategies for the synthesis of monoantennary glycans, and the optional glycoPEGylation of the same. Dark squares: GlcNAc; dark circles: Man; light circles: Gal; dark triangles: sialic acid.

Figure 11 is a scheme depicting various complex structures, which may be synthesized from an elemental trimannosyl core structure. Symbols: Squares: GlcNAc; light circles: Man; dark circles: Gal; triangles: NeuAc; diamonds: fucose; FT and FucT: fucosyltransferase; GalT: galactosyltransferase; ST: sialyltransferase; Le: Lewis antigen; SLe: sialylated Lewis antigen.

Figure 12 is an exemplary scheme for preparing O-linked glycopeptides originating with serine or threonine. Optionally, a water soluble polymer (WSP) such as poly(ethylene glycol) is added to the final glycan structure.

Figure 13 is a series of diagrams depicting the four types of O-glycan structures, termed cores 1 through 4. The core structure is outlined in dotted lines.

Figure 14, comprising Figure 14A and Figure 14B, is a series of schemes showing an exemplary embodiment of the invention in which carbohydrate residues comprising complex

carbohydrate structures and/or high mannose high mannose structures are trimmed back to the first generation biantennary structure. Optionally, fucose is added only after reaction with GnT I. A modified sugar bearing a water-soluble polymer (WSP) is then conjugated to one or more of the sugar residues exposed by the trimming back process.

Figure 15 is a scheme similar to that shown in Figure 4, in which a high mannose or complex structure is "trimmed back" to the mannose beta-linked core and a modified sugar bearing a water soluble polymer is then conjugated to one or more of the sugar residues exposed by the trimming back process. Sugars are added sequentially using glyeosyltransferases.

Figure 16 is a scheme similar to that shown in Figure 4, in which a high mannose or complex structure is trimmed back to the GlcNAc to which the first mannose is attached, and a modified sugar bearing a water soluble polymer is then conjugated to one or more of the sugar residues exposed by the trimming back process. Sugars are added sequentially using glycosyltransferases.

Figure 17 is a scheme similar to that shown in Figure 4, in which a high mannose or cpomplex structure is trimmed back to the first GlcNAc attached to the Asn of the peptide, following which a water soluble polymer is conjugated to one or more sugar residues which have subsequently been added on. Sugars are added sequentially using glycosyltransferases.

Figure 18, comprising Figure 18A and 18B, is a scheme in which an N-linked carbohydrate is optionally trimmed back from a high mannose or cpmplex structure, and subsequently derivatized with a modified sugar moiety (Gal or GlcNAc) bearing a water-soluble polymer.

Figure 19, comprising Figure 19A and 19B, is a scheme in which an N-linked carbohydrate is trimmed back from a high mannose or complex structure and subsequently derivatized with a sialic acid moiety bearing a water-soluble polymer. Sugars are added sequentially using glycosyltransferases.

Figure 20 is a scheme in which an N-linked carbohydrate is optionally trimmed back from a high mannose oor complex structure and subsequently derivatized with one or more sialic acid moieties, and terminated with a sialic acid derivatized with a water-soluble polymer. Sugars are added sequentially using glycosyltransferases.

Figure 21 is a scheme in which an O-linked saccharide is "trimmed back" and subsequently conjugated to a modified sugar bearing a water-soluble polymer. In the exemplary scheme, the carbohydrate moiety is "trimmed back" to the first generation of the biantennary structure.

Figure 22 is an exemplary scheme for trimming back the carbohydrate moiety of an O-linked glycopeptide to produce a mannose available for conjugation with a modified sugar having a water-soluble polymer attached thereto.

Figure 23, comprising Figure 23A to Figure 23C, is a series of exemplary schemes. Figure 23A is a scheme that illustrates addition of a PEGylated sugar, followed by the addition of a non-modified sugar. Figure 23B is a scheme that illustrates the addition of more that one kind of modified sugar onto one glycan. Figure 23C is a scheme that illustrates the addition of different modified sugars onto O-linked glycans and N-linked glycans.

Figure 24 is a diagram of various methods of improving the therapeutic function of a peptide by glycan remodeling, including conjugation.

Figure 25 is a set of schemes for glycan remodeling of a therapeutic peptide to treat Gaucher Disease.

Figure 26 is a scheme for glycan remodeling to generate glycans having a terminal mannose-6-phosphate moiety.

Figure 27 is a diagram illustrating the array of glycan structures found on CHO-produced glucocerebrosidase (Cerezyme™) after sialylation.

Figure 28, comprising Figure 28A to Figure 28Z and Figure 28AA to Figure 28CC, is a list of peptides useful in the methods of the invention.

Figure 29, comprising Figures 29A to 29G, provides exemplary schemes for remodeling glycan structures on granulocyte colony stimulating factor (G-CSF). Figure 29A is a diagram depicting the G-CSF peptide indicating the amino acid residue to which a glycan is bonded, and an exemplary glycan formula linked thereto. Figure 29B to 29G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 29A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 30, comprising Figures 30A to 30EE sets forth exemplary schemes for remodeling glycan structures on interferon-alpha. Figure 30A is a diagram depicting the interferon-alpha isoform 14c peptide indicating the amino acid residue to which a glycan is

bonded, and an exemplary glycan formula linked thereto. Figure 30B to 30D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30E is a diagram depicting the interferon-alpha isoform 14c peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 30F to 30N are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30O is a diagram depicting the interferon-alpha isoform 2a or 2b peptides indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 30P to 30W are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 30O based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30X is a diagram depicting the interferon-alpha-mucin fusion peptides indicating the residue(s) which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 30Y to 30AA are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 30X based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 30BB is a diagram depicting the interferon-alpha-mucin fusion peptides and interferon-alpha peptides indicating the residue(s) which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 30CC to 30EE are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 30BB based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 31, comprising Figures 31A to 31S, sets forth exemplary schemes for remodeling glycan structures on interferon-beta. Figure 31A is a diagram depicting the interferon-beta peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 31B to 31O are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 31A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 31P is a diagram depicting the interferon-beta peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 31Q to 31S are diagrams of

contemplated remodeling steps of the glycan of the peptide in Figure 31P based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 32, comprising Figures 32A to 32D, sets forth exemplary schemes for remodeling glycan structures on Factor VII and Factor VIIa. Figure 32A is a diagram depicting the Factor-VII and Factor-VIIa peptides A (solid line) and B (dotted line) indicating the residues whieh bind to glycans contemplated for remodeling, and the formulas for the glycans. Figure 32B to 32D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 32A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 33, comprising Figures 33A to 33G, sets forth exemplary schemes for remodeling glycan structures on Factor IX. Figure 33A is a diagram depicting the Factor-IX peptide indicating residues which bind to glycans contemplated for remodeling, and formulas of the glycans. Figure 33B to 33G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 33A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 34, comprising Figures 34A to 34J, sets forth exemplary schemes for remodeling glycan structures on follicle stimulating hormone (FSH), comprising α and β subunits. Figure 34A is a diagram depicting the Follicle Stimulating Hormone peptides FSHα and FSHβ indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 34B to 34J are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 34A based on the type of cell the peptides are expressed in and the desired remodeled glycan structures.

Figure 35, comprising Figures 35A to 35AA, sets forth exemplary schemes for remodeling glycan structures on Erythropoietin (EPO). Figure 35A is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35B to 35S are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 35T is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35U to 35W are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35T based on the type of cell the peptide is

expressed in and the desired remodeled glycan structure. Figure 35X is a diagram depicting the EPO peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 35Y to 35AA are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 35X based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 36, comprising Figures 36A to 36K sets forth exemplary schemes for remodeling glycan structures on Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). Figure 36A is a diagram depicting the GM-CSF peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 36B to 36G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 36A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 36H is a diagram depicting the GM-CSF peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 36I to 36K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 36H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 37, comprising Figures 37A to 37N, sets forth exemplary schemes for remodeling glycan structures on interferon-gamma. Figure 37A is a diagram depicting an interferon-gamma peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 37B to 37G are diagrams of contemplated remodeling steps of the peptide in Figure 37A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 37H is a diagram depicting an interferon-gamma peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 37I to 37N are diagrams of contemplated remodeling steps of the peptide in Figure 37H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 38, comprising Figures 38A to 38N, sets forth exemplary schemes for remodeling glycan structures on α_1-antitrypsin (ATT, or α-1 protease inhibitor). Figure 38A is a diagram depicting an AAT peptide indicating the residues which bind to glyeans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38B to 38F are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure

38A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 38G is a diagram depicting an AAT peptide indicating the residues whieh bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38H to 38J are diagrams of contemplated remodeling steps of the peptide in Figure 38G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 38K is a diagram depicting an AAT peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 38L to 38N are diagrams of contemplated remodcling steps of the peptidc in Figure 38K based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 39, comprising Figures 39A to 39J sets forth exemplary schemes for remodeling glycan structures on glucocerebrosidase. Figure 39A is a diagram depicting the glucocerebrosidase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 39B to 39F are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 39A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 39G is a diagram depicting the glucocerebrosidase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 39H to 39K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 39G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 40, comprising Figures 40A to 40W, sets forth exemplary schemes for remodeling glycan structures on Tissue-Type Plasminogen Activator (TPA). Figure 40A is a diagram depicting the TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40B to 40G are diagrams of contemplated remodeling steps of the peptide in Figure 40A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40H is a diagram depicting the TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40I to 40K are diagrams of contemplated remodeling steps of the peptide in Figure 40H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40L is a diagram depicting a

mutant TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and the formula for the glycans. Figure 40M to 40O are diagrams of contemplated remodeling steps of the peptide in Figure 40L based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40P is a diagram depicting a mutant TPA peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 40Q to 40S are diagrams of contemplated remodeling steps of the peptide in Figure 40P based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 40T is a diagram depicting a mutant TPA peptide indicating the residues which links to glycans contemplated for remodeling, and formulas for the glycans. Figure 40U to 40W are diagrams of contemplated remodeling steps of the peptide in Figure 40T based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 41, comprising Figures 41A to 41G, sets forth exemplary schemes for remodeling glycan structures on Interleukin-2 (IL-2). Figure 41A is a diagram depicting the Interleukin-2 peptide indicating the amino acid residue to which a glycan is linked, and an exemplary glycan formula linked thereto. Figure 41B to 41G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 41A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 42, comprising Figures 42A to 42M, sets forth exemplary schemes for remodeling glycan structures on Factor VIII. Figure 42A are the formulas for the glycans that bind to the N-linked glycosylation sites (A and A') and to the O-linked sites (B) of the Factor VIII peptides. Figure 42B to 42F are diagrams of contemplated remodeling steps of the peptides in Figure 42A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 42G are the formulas for the glycans that bind to the N-linked glycosylation sites (A and A') and to the O-linked sites (B) of the Factor VIII peptides. Figure 42H to 42M are diagrams of contemplated remodeling steps of the peptides in Figure 42G based on the type of cell the peptide is expressed in and the desired remodeled glycan structures.

Figure 43, comprising Figures 43A to 43L, sets forth exemplary schemes for remodeling glycan structures on urokinase. Figure 43A is a diagram depicting the urokinase peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an

exemplary glyean formula linked thereto. Figure 43B to 43F are diagrams of contemplated remodeling steps of the peptide in Figure 43A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 43G is a diagram depieting the urokinase peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 43H to 43L are diagrams of contemplated remodeling steps of the peptide in Figure 43G based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 44, comprising Figures 44A to 44J, sets forth exemplary schemes for remodeling glycan structures on human DNase (hDNase). Figure 44A is a diagram depicting the human DNase peptide indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 44B to 44F are diagrams of contemplated remodeling steps of the peptide in Figure 44A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 44G is a diagram depicting the human DNase peptide indicating residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 44H to 44J are diagrams of contemplated remodeling steps of the peptide in Figure 44F based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 45, comprising Figures 45A to 45L, sets forth exemplary schemes for remodeling glycan structures on insulin. Figure 45A is a diagram depicting the insulin peptide mutated to contain an N glycosylation site and an exemplary glycan formula linked thereto. Figure 45B to 45D are diagrams of contemplated remodeling steps of the peptide in Figure 45A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 45E is a diagram depicting insulin-mucin fusion peptides indicating a residue(s) which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 45F to 45H are diagrams of contemplated remodeling steps of the peptide in Figure 45E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 45I is a diagram depicting the insulin-mucin fusion peptides and insulin peptides indicating a residue(s) which is linked to a glycan contemplated for remodeling, and formulas for the glycan. Figure 45J to 45L are diagrams of contemplated remodeling steps of the peptide in Figure 45I based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 46, comprising Figures 46A to 46K, sets forth exemplary schemes for remodeling glycan structures on the M-antigen (preS and S) of the Hepatitis B surface protein (HbsAg). Figure 46A is a diagram depicting the M-antigen peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 46B to 46G are diagrams of contemplated remodeling steps of the peptide in Figure 46A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 46H is a diagram depicting the M-antigen peptide indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. Figure 46I to 46K are diagrams of contemplated remodeling steps of the peptide in Figure 46H based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 47, comprising Figures 47A to 47K, sets forth exemplary schemes for remodeling glycan structures on human growth hormone, including N, V and variants thereof. Figure 47A is a diagram depicting the human growth hormone peptide indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 47B to 47D are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 47A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 47E is a diagram depicting the three fusion peptides comprising the human growth hormone peptide and part or all of a mucin peptide, and indicating a residue(s) which is linked to a glycan contemplated for remodeling, and exemplary glycan formula(s) linked thereto. Figure 47F to 47K are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 47E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 48, comprising Figures 48A to 48G, sets forth exemplary schemes for remodeling glycan structures on a TNF Receptor-IgG Fc region fusion protein (Enbrel™). Figure 48A is a diagram depicting a TNF Receptor--IgG Fc region fusion peptide which may be mutated to contain additional N-glycosylation sites indicating the residues which bind to glycans contemplated for remodeling, and formulas for the glycans. The TNF receptor peptide is depicted in bold line, and the IgG Fc regions is depicted in regular line. Figure 48B to 48G are diagrams of contemplated remodeling steps of the peptide in Figure 48A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 49, comprising Figures 49A to 49D, sets forth exemplary schemes for remodeling glycan structures on an anti-HER2 monoclonal antibody (Herceptin™). Figure 49A is a diagram depicting an anti-HER2 monoclonal antibody which has been mutated to contain an N-glycosylation site(s) indicating a residue(s) on the antibody heavy chain which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 49B to 49D are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 49A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 50, comprising Figures 50A to 50D, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to Protein F of Respiratory Syncytial Virus (Synagis™). Figure 50A is a diagram depicting a monoclonal antibody to Protein F peptide which is mutated to contain an N-glycosylation site(s) indicating a residue(s) which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 50B to 50D are diagrams of contemplated remodeling steps of the peptide in Figure 50A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 51, comprising Figures 51A to 51D, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to TNF-α (Remicade™). Figure 51A is a diagram depicting a monoclonal antibody to TNF-α which has an N-glycosylation site(s) indicating a residue which is linked to a glycan contemplated for remodeling, and an exemplary glycan formula linked thereto. Figure 51B to 51D are diagrams of contemplated remodeling steps of the peptide in Figure 51A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 52, comprising Figures 52A to 52L, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to glycoprotein IIb/IIIa (Reopro™). Figure 52A is a diagram depicting a mutant monoclonal antibody to glycoprotein IIb/IIIa peptides which have been mutated to contain an N-glycosylation site(s) indicating the residue(s) which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52B to 52D are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 52E is a diagram depicting monoclonal antibody to glycoprotein IIb/IIIa-

mucin fusion peptides indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52F to 52H are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 52I is a diagram depicting monoclonal antibody to glycoprotein IIb/IIIa- mucin fusion peptides and monoclonal antibody to glycoprotein IIb/IIIa peptides indicating the residues which bind to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 52J to 52L are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 53, comprising Figures 53A to 53G, sets forth exemplary schemes for remodeling glycan structures on a monoclonal antibody to CD20 (Rituxan™). Figure 53A is a diagram depicting monoclonal antibody to CD20 which have been mutated to contain an N-glycosylation site(s) indicating the residue which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 53B to 53D are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 53A based on the type of cell the peptide is expressed in and the desired remodeled glycan structure. Figure 53E is a diagram depicting monoclonal antibody to CD20 which has been mutated to contain an N-glycosylation site(s) indicating the residue(s) which is linked to glycans contemplated for remodeling, and exemplary glycan formulas linked thereto. Figure 53F to 53G are diagrams of contemplated remodeling steps of the glycan of the peptides in Figure 53E based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 54, comprising Figures 54A to 54O, sets forth exemplary schemes for remodeling glycan structures on anti-thrombin III (AT III). Figure 54A is a diagram depicting the anti-thrombin III peptide indicating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54B to 54G are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54A based on the type of cell the peptide is exprcssed in and the desired remodeled glycan structure. Figure 54H is a diagram depicting the anti-thrombin III peptide indicating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54I to 54K are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54H based on the type of cell the peptide is expressed in and

the desired remodeled glycan structure. Figure 54L is a diagram depicting the anti-thrombin III peptide indieating the amino acid residues to which an N-linked glycan is linked, and an exemplary glycan formula linked thereto. Figure 54M to 54O are diagrams of contemplated remodeling steps of the glycan of the peptide in Figure 54L based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 55, comprising Figures 55A to 55J, sets forth exemplary schemes for remodeling glycan structures on subunits α and β of human Chorionic Gonadotropin (hCG). Figure 55A is a diagram depicting the hCGα and hCGβ peptides indicating the residues which bind to N-linked glycans (A) and O-linked glycans (B) contemplated for remodeling, and formulas for the glycans. Figure 55B to 55J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glyean structure.

Figure 56, comprising Figures 56A to 56J, sets forth exemplary schemes for remodeling glycan structures on alpha-galactosidase (Fabrazyme™). Figure 56A is a diagram depicting the alpha-galactosidase A peptide indicating the amino acid residues which bind to N-linked glycans (A) contemplated for remodeling, and formulas for the glycans. Figure 56B to 56J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 57, comprising Figures 57A to 57J, sets forth exemplary schemes for remodeling glycan structures on alpha-iduronidase (Aldurazyme™). Figure 57A is a diagram depicting the alpha-iduronidase peptide indicating the amino acid residues which bind to N-linked glycans (A) contemplated for remodeling, and formulas for the glycans. Figure 57B to 57J are diagrams of contemplated remodeling steps based on the type of cell the peptide is expressed in and the desired remodeled glycan structure.

Figure 58, comprising Figures 58A and 58B, is an exemplary nucleotide and corresponding amino acid sequence of granulocyte colony stimulating factor (G-CSF) (SEQ ID NOS: 1 and 2, respectively).

Figure 59, comprising Figures 59A through 59D, is an exemplary nucleotide and corresponding amino acid sequence of interferon alpha (IFN-alpha) (Figures 59A and 59 B, SEQ ID NOS: 3 and 4, respectively), and an exemplary nucleotide and corresponding amino

acid sequence of interferon-omega (IFN-omega) (Figures 59C and 59 D, SEQ ID NOS: 74 and 75, respectively).

Figure 60, comprising Figures 60A and 60B, is an exemplary nucleotide and corresponding amino acid sequence of interferon beta (IFN-beta) (SEQ ID NOS: 5 and 6, respectively).

Figure 61, comprising Figures 61A and 61B, is an exemplary nucleotide and corresponding amino acid sequence of Factor VIIa (SEQ ID NOS: 7 and 8, respectively).

Figure 62, comprising Figures 62A and 62B, is an exemplary nucleotide and corresponding amino acid sequence of Factor IX (SEQ ID NOS: 9 and 10, respectively).

Figure 63, comprising Figures 63A through 63D, is an exemplary nucleotide and corresponding amino acid sequence of the alpha and beta chains of follicle stimulating hormone (FSH), respectively (SEQ ID NOS: 11 through 14, respectively).

Figure 64, comprising Figures 64A and 64B, is an exemplary nucleotide and corresponding amino acid sequence of erythropoietin (EPO) (SEQ ID NOS: 15 and 16, respectively).

Figure 65 is an amino acid sequence of mature EPO, i.e. 165 amino acids (SEQ ID NO:73).

Figure 66, comprising Figures 66A and 66B, is an exemplary nucleotide and corresponding amino acid sequence of granulocyte-macrophage colony stimulating factor (GM-CSF) (SEQ ID NOS: 17 and 18, respectively).

Figure 67, comprising Figures 67A and 67B, is an exemplary nucleotide and corresponding amino acid sequence of interferon gamma (IFN-gamma) (SEQ ID NOS: 19 and 20, respectively).

Figure 68, comprising Figures 68A and 68B, is an exemplary nucleotide and corresponding amino acid sequence of α-1-protease inhibitor (A-1-PI, or α-antitrypsin) (SEQ ID NOS: 21 and 22, respectively).

Figure 69, comprising Figures 69A-1 to 69A-2, and 69B, is an exemplary nucleotide and corresponding amino acid sequence of glucocerebrosidase (SEQ ID NOS: 23 and 24, respectively).

Figure 70, comprising Figures 70A and 70B, is an exemplary nucleotide and corresponding amino acid sequence of tissue-type plasminogen activator (TPA) (SEQ ID NOS: 25 and 26, respectively).

Figure 71, comprising Figures 71A and 71B, is an exemplary nucleotide and corresponding amino acid sequence of Interleukin-2 (IL-2) (SEQ ID NOS: 27 and 28, respectively).

Figure 72, comprising Figures 72A-1 through 72A-4 and Figure 72B-1 through 72B-4, is an exemplary nucleotide and corresponding amino acid sequence of Factor VIII (SEQ ID NOS: 29 and 30, respectively).

Figure 73, comprising Figures 73A and 73B, is an exemplary nucleotide and corresponding amino acid sequence of urokinase (SEQ ID NOS: 33 and 34, respectively).

Figure 74, comprising Figures 74A and 74B, is an exemplary nucleotide and corresponding amino acid sequence of human recombinant DNase (hrDNase) (SEQ ID NOS: 39 and 40, respectively).

Figure 75, comprising Figures 75A and 75B, is an exemplary nucleotide and corresponding amino acid sequence of an insulin molecule (SEQ ID NOS: 43 and 44, respectively).

Figure 76, comprising Figures 76A and 76B, is an exemplary nucleotide and corresponding amino acid sequence of S-protein from a Hepatitis B virus (HbsAg) (SEQ ID NOS: 45 and 46, respectively).

Figure 77, comprising Figures 77A and 77B, is an exemplary nucleotide and corresponding amino acid sequence of human growth hormone (hGH) (SEQ ID NOS: 47 and 48, respectively).

Figure 78, comprising Figures 78A and 78B, are exemplary nucleotide and corresponding amino acid sequences of anti-thrombin III. Figures 78A and 78B, are an exemplary nucleotide and corresponding amino acid sequences of "WT" anti-thrombin III (SEQ ID NOS: 63 and 64, respectively).

Figure 79, comprising Figures 79A to 79D, are exemplary nucleotide and corresponding amino acid sequences of human chorionic gonadotropin (hCG) α and β subunits. Figures 79A and 79B are an exemplary nucleotide and eorresponding amino acid sequence of the α-subunit of human chorionic gonadotropin (SEQ ID NOS: 69 and 70,

respectively). Figures 79C and 79D are an exemplary nucleotide and corresponding amino acid sequence of the beta subunit of human chorionic gonadotrophin (SEQ ID NOS: 71 and 72, respectively).

Figure 80, comprising Figures 80A and 80B, is an exemplary nucleotide and corresponding amino acid sequence of α-iduronidase (SEQ ID NOS: 65 and 66, respectively).

Figure 81, comprising Figures 81A and 81B, is an exemplary nucleotide and corresponding amino acid sequence of α-galactosidase A (SEQ ID NOS: 67 and 68, respectively).

Figure 82, comprising Figures 82A and 82B, is an exemplary nucleotide and corresponding amino acid sequence of the 75 kDa tumor necrosis factor receptor (TNF-R), which comprises a portion of Enbrel™ (tumor necrosis factor receptor (TNF-R)/IgG fusion) (SEQ ID NOS: 31 and 32, respectively).

Figure 83, comprising Figures 83A and 83B, is an exemplary amino acid sequence of the light and heavy chains, respectively, of Herceptin™ (monoclonal antibody (MAb) to Her-2, human epidermal growth factor receptor) (SEQ ID NOS: 35 and 36, respectively).

Figure 84, comprising Figures 84A and 84B, is an exemplary amino acid sequence the heavy and light chains, respectively, of Synagis™ (MAb to F peptide of Respiratory Syncytial Virus) (SEQ ID NOS: 37 and 38, respectively).

Figure 85, comprising Figures 85A and 85B, is an exemplary nucleotide and corresponding amino acid sequence of the non-human variable regions of Remicade™ (MAb to TNFα) (SEQ ID NOS: 41 and 42, respectively).

Figure 86, comprising Figures 86A and 86B, is an exemplary nucleotide and corresponding amino acid sequence of the Fc portion of human IgG (SEQ ID NOS: 49 and 50, respectively).

Figure 87 is an exemplary amino acid sequence of the mature variable region light chain of an anti-glycoprotein IIb/IIIa murine antibody (SEQ ID NO: 52).

Figure 88 is an exemplary amino acid sequence of the mature variable region heavy chain of an anti-glycoprotein IIb/IIIa murine antibody (SEQ ID NO: 54).

Figure 89 is an exemplary amino acid sequence of variable region light chain of a human IgG (SEQ ID NO: 51).

Figure 90 is an exemplary amino acid sequence of variable region heavy chain of a human IgG (SEQ ID NO:53).

Figure 91 is an exemplary amino acid sequence of a light chain of a human IgG (SEQ ID NO:55).

Figure 92 is an exemplary amino acid sequence of a heavy chain of a human IgG (SEQ ID NO:56).

Figure 93, comprising Figures 93A and 93B, is an exemplary nucleotide and eorresponding amino acid sequence of the mature variable region of the light chain of an anti-CD20 murine antibody (SEQ ID NOS: 59 and 60, respectively).

Figure 94, comprising Figures 94A and 94B, is an exemplary nucleotide and corresponding amino acid sequence of the mature variable region of the heavy chain of an anti-CD20 murine antibody (SEQ ID NOS: 61 and 62, respectively).

Figure 95, comprising Figures 95A through 95E, is the nucleotide sequence of the tandem chimeric antibody expression vector TCAE 8 (SEQ ID NO:57).

Figure 96, comprising Figures 96A through 96E, is the nucleotide sequence of the tandem chimeric antibody expression vector TCAE 8 containing the light and heavy variable domains of the anti-CD20 murine antibody (SEQ ID NO:58).

Figure 97, comprising Figures 97A to 97C, are graphs depicting 2-AA HPLC analysis of glycans released by PNGaseF from myeloma-expressed Cri-IgG1 antibody. The structure of the glycans is determined by retention time: the G0 glycoform elutes at 30 min., the G1 glycoform elutes at ~ 33 min., the G2 glycoform elutes at about approximately 37 min. and the S1-G1 glycoform elutes at ~ 70 min. Figure 97A depicts the analysis of the DEAE antibody sample. Figure 97B depicts the analysis of the SPA antibody sample. Figure 97C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 14.

Figure 98, comprising Figures 98A to 98C, are graphs depicting the MALDI analysis of glycans released by PNGaseF from myeloma-expressed Cri-IgG1 antibody. The glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 98A depicts the analysis of the DEAE antibody sample. Figure 98B depicts the analysis of the SPA antibody sample. Figure 98C depicts the analysis of the Fc antibody sample.

Figure 99, comprising Figures 99A to 99D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 99A. Figure 99B depicts the analysis of the DEAE antibody sample. Figure 99C depicts the analysis of the SPA antibody sample. Figure 99D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 15.

Figure 100, comprising Figures 100A to 100D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 100A. Figure 100B depicts the analysis of the DEAE antibody sample. Figure 100C depicts the analysis of the SPA antibody sample. Figure 100D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 16.

Figure 101, comprising Figures 101A to 101C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. The released glycans were labeled with 2AA and separated by HPLC on a NH2P-50 4D amino column. Figure 101A depicts the analysis of the DEAE antibody sample. Figure 101B depicts the analysis of the SPA antibody sample. Figure 101C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 16

Figure 102, comprising Figures 102A to 102C, are graphs depicting the MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G0 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 102A depicts the analysis of the DEAE antibody sample. Figure 102B depicts the analysis of the SPA antibody sample. Figure 102C depicts the analysis of the Fc antibody sample.

Figure 103, comprising Figures 103A to 103D, are graphs depicting the capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 103A. Figure 103B

depicts the analysis of the DEAE antibody sample. Figure 103C depicts the analysis of the SPA antibody sample. Figure 103D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 17.

Figure 104, comprising Figures 104A to 104C, are graphs depicting the 2-AA HPLC analysis of glyeans released from remodeled Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. The released glycans were labeled with 2AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 104A depicts the analysis of the DEAE antibody sample. Figure 104B depicts the analysis of the SPA antibody sample. Figure 104C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 17.

Figure 105, comprising Figures 105A to 105C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled to contain G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 105A depicts the analysis of the DEAE antibody sample. Figure 105B depicts the analysis of the SPA antibody sample. Figure 105C depicts the analysis of the Fc antibody sample.

Figure 106, comprising Figures 106A to 106D, are graphs depicting capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I treatment of M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 106A. Figure 106B depicts the analysis of the DEAE antibody sample. Figure 106C depicts the analysis of the SPA antibody sample. Figure 106D depicts the analysis of the Fc antibody sample.

Figure 107, comprising Figures 107A to 107C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been remodeled by GnT-I treatment of M3N2 glycoforms. The released glycans were labeled with 2-AA and separated by HPLC on a NH2P-50 4D amino column. Figure 107A depicts the analysis of the DEAE antibody sample. Figure 107B depicts the analysis of the SPA antibody sample. Figure 107C depicts the analysis of the Fc antibody sample.

Figure 108, comprising Figures 108A to 108C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I

treatment of M3N2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 108A depicts the analysis of the DEAE antibody sample. Figure 108B depiets the analysis of the SPA antibody sample. Figure 108C depicts the analysis of the Fe antibody sample.

Figure 109, comprising Figures 109A to 109D, are graphs depicting capillary electrophoresis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I, II and III treatment of M3N2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 109A. Figure 109B depicts the analysis of the DEAE antibody sample. Figure 109C depicts the analysis of the SPA antibody sample. Figure 109D depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 18.

Figure 110, comprising Figures 110A to 110C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by GnT-I, II and III treatment of M3N2 glycoforms. The released glycans were labeled with 2AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 110A depicts the analysis of the DEAE antibody sample. Figure 110B depicts the analysis of the SPA antibody sample. Figure 110C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 18.

Figure 111, comprising Figures 111A to 111C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by galactosyltransferase treatment of NGA2F glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 111A depicts the analysis of the DEAE antibody sample. Figure 111B depicts the analysis of the SPA antibody sample. Figure 111C depicts the analysis of the Fc antibody sample.

Figure 112, comprising 112A to 112D, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies containing NGA2F isoforms before GalT1 treatment (Figures 112A and 112C) and after GalT1 treatment (Figures 112B and 112D). Figures 112A and 112B depict the analysis of the DEAE sample of antibodies. Figures 112C and 112D depict the analysis of the Fc sample of antibodies. The released glycans were labeled with 2AA and separated by HPLC on a NH2P-50 4D amino column.

Figure 113, comprising 113A to 113C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that have been glycoremodeled by ST3Gal3 treatment of G2 glycoforms. The released glycans are labeled with 2-AA and then separated by HPLC on a NH2P-50 4D amino column. Figure 113A depicts the analysis of the DEAE antibody sample. Figure 113B depicts the analysis of the SPA antibody sample. Figure 113C depicts the analysis of the Fc antibody sample. The percent area under the peaks for these graphs is summarized in Table 19.

Figure 114, comprising Figures 114A to 114C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST3Gal3 treatment of G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 114A depicts the analysis of the DEAE antibody sample. Figure 114B depicts the analysis of the SPA antibody sample. Figure 114C depicts the analysis of the Fc antibody sample.

Figure 115, comprising Figures 115A to 115D, are graphs depicting capillary electrophoresis analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. A graph depicting the capillary electrophoresis analysis of glycan standards derivatized with APTS is shown in Figure 115A. Figure 115B depicts the analysis of the DEAE antibody sample. Figure 115C depicts the analysis of the SPA antibody sample. Figure 115D depicts the analysis of the Fc antibody sample.

Figure 116, comprising Figures 116A to 116C, are graphs depicting 2-AA HPLC analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. The released glycans were labeled with 2-AA and separated by HPLC on a NH2P-50 4D amino column. Figure 116A depicts the analysis of the DEAE antibody sample. Figure 116B depicts the analysis of the SPA antibody sample. Figure 116C depicts the analysis of the Fc antibody sample.

Figure 117, comprising Figures 117A to 117C, are graphs depicting MALDI analysis of glycans released from Cri-IgG1 antibodies that had been glycoremodeled by ST6Gal1 treatment of G2 glycoforms. The released glycans were derivatized with 2-AA and then analyzed by MALDI. Figure 117A depicts the analysis of the DEAE antibody sample.

Figure 117B depicts the analysis of the SPA antibody sample. Figure 117C depicts the analysis of the Fc antibody sample.

Figure 118, comprising Figures 118A to 118E, depicts images of SDS-PAGE analysis of the glycoremodeled of Cri-IgG1 antibodies with different glycoforms under non-reducing conditions. Bovine serum albumin (BSA) was run under reducing conditions as a quantitative standard. Protein molecular weight standards are displayed and their size is indicated in kDa. Figure 118A depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain G0 and G2 glycoforms. Figure 118B depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain NGA2F (bisecting) and GnT-I-M3N2 (GnT1) glycoforms. Figure 118C depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain S2G2 (ST6Gal1) glycoforms. Figure 118D depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain M3N2 glycoforms, and BSA. Figure 118E depicts SDS-PAGE analysis of the DEAE, SPA and Fc Cri-IgG1 antibodies glycoremodeled to contain Gal-NGA2F (Gal-bisecting) glycoforms, and BSA.

Figure 119 is an image of an acrylamide gel depicting the results of FACE analysis of the pre- and post-sialylation of TP10. The $BiNA_0$ species has no sialic acid residues. The $BiNA_1$ species has one sialic acid residue. The $BiNA_2$ species has two sialic acid residues. Bi = biantennary; NA = neuraminic acid.

Figure 120 is a graph depicting the plasma concentration in μg/ml over time of pre- and post-sialylation TP10 injected into rats.

Figure 121 is a graph depicting the area under the plasma concentration-time curve (AUC) in μg/hr/ml for pre- and post sialylated TP10.

Figure 122 is an image of an acrylamide gel depicting the results of FACE glycan analysis of the pre- and post-fucosylation of TP10 and FACE glycan analysis of CHO cell produced TP-20. The $BiNA_2F_2$ species has two neuraminic acid (NA) residues and two fucose residues (F).

Figure 123 is a graph depicting the *in vitro* binding of TP20 ($sCR1sLe^X$) glycosylated *in vitro* (diamonds) and *in vivo* in Lec11 CHO cells (squares).

Figure 124 is a graph depicting the analysis by 2-AA HPLC of glyeoforms from the GlcNAc-ylation of EPO.

Figure 125, comprising Figures 125A and 125B, are graphs depicting the 2-AA HPLC analysis of two lots of EPO to which N-acetylglucosamine was been added. Figure 125A depicts the analysis of lot A, and Figure 125B depicts the analysis of lot B.

Figure 126 is a graph depicting the 2-AA HPLC analysis of the products the reaction introducing a third glycan branch to EPO with GnT-V.

Figure 127 is a graph depicting a MALDI-TOF spectrum of the glycans of the EPO preparation after treatment with GnT-I, GnT-II, GnT-III, GnT-V and GalT1, with appropriate donor groups.

Figure 128 is a graph depicting a MALDI spectrum the glycans of native EPO.

Figure 129 is an image of an SDS-PAGE gel of the products of the PEGylation reactions using CMP-SA-PEG (1 kDa), and CMP-SA-PEG (10 kDa).

Figure 130 is a graph depicting the results of the *in vitro* bioassay of PEGylated EPO. Diamonds represent the data from sialylated EPO having no PEG molecules. Squares represent the data obtained using EPO with PEG (1 kDa). Triangles represent the data obtained using EPO with PEG (10 kDa).

Figure 131 is a diagram of CHO-expressed EPO. The EPO polypeptide is 165 amino acids in length, with a molecular weight of 18 kDa without glycosylation. The glycosylated forms of EPO produced in CHO cells have a molecular weight of about 33 kDa to 39 kDa. The shapes which represent the sugars in the glycan chains are identified in the box at the lower edge of the drawing.

Figure 132 is a diagram of insect cell expressed EPO. The shapes that represent the sugars in the glycan chains are identified in the box at the lower edge of FIG. 131.

Figure 133 is a bar graph depicting the molecular weights of the EPO peptides expressed in insect cells which were remodeled to form complete mono-, bi- and tri-antennary glycans, with optional glycoPEGylation with 1 kDa, 10 kDa or 20 kDa PEG. Epoetin™ is EPO expressed in mammalian cells without further glycan modification or PEGylation. NESP (Aranesp™, Amgen, Thousand Oaks, CA) is a form of EPO having 5 N-linked glycan sites that is also expressed in mammalian cells without further glycan modification or PEGylation.

Figure 134, comprising Figures 134A and 134B, depicts one scheme for the remodeling and glycoPEGylation of insect cell expressed EPO. Figure 134A depicts the

remodeling and glycoPEGylation steps that remodel the insect expressed glycan to a mono-antennary glycoPEGylated glycan. Figure 134B depicts the remodeled EPO polypeptide having a completed glycoPEGylated mono-antennary glycan at each N-linked glyean site of the polypeptide. The shapes that represent the sugars in the glycan chains are identified in the box at the lower edge of FIG. 131, except that the triangle represents sialic acid.

Figure 135 is a graph depicting the *in vitro* bioactivities of EPO-SA and EPO-SA-PEG constructs. The *in vitro* assay measured the proliferation of TF-1 erythroleukemia cells which were maintained for 48 hr in RBMI + FBS 10% + GM-CSF (12 ng/ml) after the EPO construct was added at 10.0, 5.0, 2.0, 1.0, 0.5, and 0 μg/ml. Tri-SA refers to EPO constructs where the glycans are tri-antennary and have SA. Tri-SA 1K PEG refers to EPO constructs where the glycans are tri-antennary and have Gal and are then glycoPEGylated with SA-PEG 1 kDa. Di-SA 10K PEG refers to EPO constructs where the glycans are bi-antennary and have Gal and are then glycoPEGylated with SA-PEG 10 kDa. Di-SA 1K PEG refers to EPO constructs where the glycans are bi-antennary and have Gal and are then glycoPEGylated with SA-PEG 1 kDa. Di-SA refers to EPO constructs where the glycans are bi-antennary and are built out to SA. Epogen™ is EPO expressed in CHO cells with no further glycan modification.

Figure 136 is a graph depicting the pharmacokinetics of the EPO constructs in rat. Rats were bolus injected with [I^{125}]-labeled glycoPEGylated and non-glycoPEGylated EPO. The graph shows the concentration of the radio-labeled EPO in the bloodstream of the rat at 0 to about 72 minutes after injection. "Biant-10K" refers to EPO with biantennary glycan structures with terminal 10 kDa PEG moieties. "Mono-20K" refers to EPO with monoantennary glycan structures with terminal 20 kDa PEG moieties. NESP refers to the commercially available Aranesp. "Biant-1K" refers to EPO with biantennary glycan structures with terminal 1 kDa PEG moieties. "Biant-SA" refers to EPO with biantennary glycan structures with terminal 1 kDa moieties. The concentration of the EPO constructs in the bloodstream at 72 hr. is as follows: Biant-10K, 5.1 cpm/ml; Mono-20K, 3.2 cpm/ml; NESP, 1 cpm/ml; and Biant-1K, 0.2 cpm/ml; Biant-SA, 0.1 cpm/ml. The relative area under the curve of the EPO constructs is as follows: Biant-10K, 2.9; Mono-20K, 2.1; NESP, 1; Biant-1K, 0.5; and Biant-SA, 0.2.

Figure 137 is a bar graph depicting the ability of the EPO constructs to stimulate reticulocytosis *in vivo*. Each treatment group is composed of eight mice. Mice were given a single subcutaneous injection of 10 μg protein / kg body weight. The percent reticulocytosis was measured at 96 hr. Tri-antennary-SA2,3(6) construct has the SA molecule bonded in a 2,3 or 2,6 linkage (see, Example 18 herein for preparation) wherein the glycan on EPO is tri-antennary N-glycans with SA-PEG 10 K is attached thereon. Similarly, bi-antennary-10K PEG is EPO having a bi-antennary N-glycan with SA-PEG at 10 K PEG attached thereon.

Figure 138 is a bar graph depicting the ability of EPO constructs to increase the hematocrit of the blood of mice *in vivo*. CD-1 female mice were injected i.p. with 2.5 μg protein/kg body weight. The hematocrit of the mice was measured on day 15 after the EPO injection. Bi-1k refers to EPO constructs where the glycans are bi-antennary and are built out to the Gal and then glycoPEGylated with SA-PEG 1 kDa. Mono-20k refers to EPO constructs where the glycans are mono-antennary and are built out to the Gal and then glycoPEGylated with SA-PEG 20 kDa.

Figure 139, comprising Figures 139A and 139B, depicts the analysis of glycans enzymatically released from EPO expressed in insect cells (Protein Sciences, Lot # 060302). Figure 139A depicts the HPLC analysis of the released glycans. Figure 139B depicts the MALDI analysis of the released glycans. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

Figure 140 depicts the MALDI analysis of glycans released from EPO after the GnT-I/GalT-1 reaction. The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, stars represent galactose.

Figure 141 depicts the SDS-PAGE analysis of EPO after the GnT-I/GalT-1 reaction, Superdex 75 purification, ST3Gal3 reaction with SA-PEG (10 kDa) and SA-PEG (20 kDa).

Figure 142 depicts the results of the TF-1 cell *in vitro* bioassay of PEGylated mono-antennary EPO.

Figure 143, comprising Figures 143A and 143B, depicts the analysis of glycan released from EPO after the GnT-I/GnT-II reaction. Figure 143A depicts the HPLC analysis of the released glycans, where peak 3 represents the bi-antennary GlcNAc glycan. Figure

143B depicts the MALDI analysis of the released glycans. The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

Figure 144, comprising Figures 144A and 144B, depict the HPLC analysis of glycans released from EPO after the GalT-1 reaction. Figure 144A depicts the glycans released after the small scale GalT-1 reaction. Figure 144B depicts the glycans released after the large scale GalT-1 reaction. In both figures, Peak 1 is the bi-antennary glycan with terminal galactose moieties and Peak 2 is the bi-antennary glycan without terminal galactose moieties.

Figure 145 depicts the Superdex 75 chromatography separation of EPO species after the GalT-1 reaction. Peak 2 contains EPO with bi-antennary glycans with terminal galactose moieties.

Figure 146 depicts the SDS-PAGE analysis of each of the products of the glycoremodeling process to make bi-antennary glycans with terminal galactose moieties.

Figure 147 depicts the SDS-PAGE analysis of EPO after ST3Gal3 sialylation or PEGylation with SA-PEG (1 kDa) or SA-PEG (10 kDa).

Figure 148 depicts the HPLC analysis of glycans released from EPO after the GnT-I/GnT-II reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

Figure 149 depicts the HPLC analysis of glycans released from EPO after the GnT-V reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose.

Figure 150 depicts the HPLC analysis of glycans released from EPO after the GalT-1 reaction. The struetures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, open circles represent galactose and triangles represent sialic acid.

Figure 151 depicts the HPLC analysis of glycans released from EPO after the ST3Gal3 reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks. Diamonds represent fucose, and squares represent GlcNAc, circles represent mannose, open circles represent galactose and triangles represent sialic acid.

Figure 152 depicts the HPLC analysis of glycans released from EPO after the ST6Gal1 reaction. The structures of the glycans have been determined by comparison of the peak retention with that of standard glycans. The glycan structures are depicted beside the peaks.

Figure 153 depicts the results of the TF-1 cells *in vitro* bioassay of EPO with bi-antennary and triantennary glycans. "Di-SA" refers to EPO with bi-antennary glycans that terminate in sialic acid. "Di-SA 10K PEG" refers to EPO with bi-antennary glycans that terminate in sialic acid derivatized with PEG (10 kDa). "Di-SA 1K PEG" refers to EPO with bi-antennary glycans that terminate in sialic acid derivatized with PEG (1 kDa). "Tri-SA ST6 + ST3" refers to EPO with tri-antennary glycans terminating in 2,6-SA capped with 2,3-SA. "Tri-SA ST3" refers to EPO with tri-antennary glycans terminating in 2,3-SA.

Figure 154 is an image of an IEF gel depicting the pI of the products of the desialylation procedure. Lanes 1 and 5 are IEF standards. Lane 2 is Factor IX protein. Lane 3 is rFactor IX protein. Lane 4 is the desialylation reaction of rFactor IX protein at 20 hr.

Figure 155 is an image of an SDS-PAGE gel depicting the molecular weight of Factor IX conjugated with either SA-PEG (1 kDa) or SA-PEG (10 kDa) after reaction with CMP-SA-PEG. Lanes 1 and 6 are SeeBlue +2 molecular weight standards. Lane 2 is rF-IX. Lane 3 is desialylated rF-IX. Lane 4 is rFactor IX conjugated to SA-PEG (1 kDa). Lane 5 is rFactor IX conjugated to SA-PEG (10 kDa).

Figure 156 is an image of an SDS-PAGE gel depicting the reaction products of direct-sialylation of Factor-IX and sialic acid capping of Factor-IX-SA-PEG. Lane 1 is protein standards, lane 2 is blank; lane 3 is rFactor-IX; lane 4 is SA capped rFactor-IX-SA-PEG (10 kDa); lane 5 is rFactor-IX-SA-PEG (10 kDa); lane 6 is ST3Gal1; lane 7 is ST3Gal3; lanes 8, 9, 10 are rFactor-IX-SA-PEG(10 kDa) with no prior sialidase treatment.

Figure 157 is an image of an isoelectric focusing gel (pH 3-7) of asialo-Factor VIIa. Lane 1 is rFactor VIIa; lanes 2-5 are asialo-Factor VIIa.

Figure 158 is a graph of a MALDI spectra of Factor VIIa.

Figure 159 is a graph of a MALDI spectra of Factor VIIa-PEG (1 kDa).

Figure 160 is a graph depicting a MALDI spectra of Factor VIIa-PEG (10 kDa).

Figure 161 is an image of an SDS-PAGE gel of PEGylated Factor VIIa. Lane 1 is asialo-Factor VIIa. Lane 2 is the product of the reaction of asialo-Factor VIIa and CMP-SA-PEG(1 kDa) with ST3Gal3 after 48 hr. Lane 3 is the product of the reaction of asialo-Factor VIIa and CMP-SA-PEG (1 kDa) with ST3Gal3 after 48 hr. Lane 4 is the product of the reaction of asialo-Factor VIIa and CMP-SA-PEG (10 kDa) with ST3Gal3 at 96 hr.

Figure 162 is an image of an isoelectric focusing (IEF) gel depicting the products of the desialylation reaction of human pituitary FSH. Lanes 1 and 4 are isoelectric focusing (IEF) standards. Lane 2 is native FSH. Lane 3 is desialylated FSH.

Figure 163 is an image of an SDS-PAGE gel of the products of the reactions to make PEG-sialylation of rFSH. Lanes 1 and 8 are SeeBlue+2 molecular weight standards. Lane 2 is 15 μg of native FSH. Lane 3 is 15 μg of asialo-FSH (AS-FSH). Lane 4 is 15 μg of the products of the reaction of AS-FSH with CMP-SA. Lane 5 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (1 kDa). Lane 6 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (5 kDa). Lane 7 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (10 kDa).

Figure 164 is an image of an isoelectric focusing gel of the products of the reactions to make PEG-sialylation of FSH. Lanes 1 and 8 are IEF standards. Lane 2 is 15 μg of native FSH. Lane 3 is 15 μg of asialo-FSH (AS-FSH). Lane 4 is 15 μg of the products of the reaction of AS-FSH with CMP-SA. Lane 5 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (1 kDa). Lane 6 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (5 kDa). Lane 7 is 15 μg of the products of the reaction of AS-FSH with CMP-SA-PEG (10 kDa).

Figure 165 is an image of an SDS-PAGE gel of native non-recombinant FSH produced in human pituitary cells. Lanes 1, 2 and 5 are SeeBlue™+2 molecular weight standards. Lanes 3 and 4 are native FSH at 5 μg and 25 μg, respectively.

Figure 166 is an image of an isoelectric focusing gel (pH 3-7) depicting the products of the asialylation reaction of rFSH. Lanes 1 and 4 are IEF standards. Lane 2 is native rFSH. Lane 3 is asialo-rFSH.

Figure 167 is an image of an SDS-PAGE gel depicting the results of the PEG-sialylation of asialo-rFSH. Lane 1 is native rFSH. Lane 2 is asialo-FSH. Lane 3 is the products of the reaction of asialo-FSH and CMP-SA. Lanes 4-7 are the products of the reaction between asialoFSH and 0.5 mM CMP-SA-PEG (10 kDa) at 2 hr, 5 hr, 24 hr, and 48 hr, respectively. Lane 8 is the products of the reaction between asialo-FSH and 1.0 mM CMP-SA-PEG (10 kDa) at 48 hr. Lane 9 is the products of the reaction between asialo-FSH and 1.0 mM CMP-SA-PEG (1 kDa) at 48 hr.

Figure 168 is an image of an isoelectric focusing gel showing the products of PEG-sialylation of asialo-rFSH with a CMP-SA-PEG (1 kDa). Lane 1 is native rFSH. Lane 2 is asialo-rFSH. Lane 3 is the products of the reaction of asialo-rFSH and CMP-SA at 24 hr. Lanes 4-7 are the products of the reaction of asialo-rFSH and 0.5 mM CMP-SA-PEG (1 kDa) at 2 hr, 5 hr, 24 hr, and 48 hr, respectively. Lane 8 is blank. Lanes 9 and 10 are the products of the reaction at 48 hr of asialo-rFSH and CMP-SA-PEG (10 kDa) at 0.5 mM and 1.0 mM, respectively.

Figure 169 is graph of the pharmacokinetics of rFSH and rFSH-SA-PEG (1 kDa and 10 kDa). This graph illustrates the relationship between the time a rFSH compound is in the blood stream of the rat, and the mean concentration of the rFSH compound in the blood for glycoPEGylated rFSH as compared to non-PEGylated rFSH.

Figure 170 is a graph of the results of the FSH bioassay using Sertoli cells. This graph illustrates the relationship between the FSH concentration in the Sertoli cell incubation medium and the amount of 17-β estradiol released from the Sertoli cells.

Figure 171 is a graph depicting the results of the Steelman-Pohley bioassay of glycoPEGylated and non-glycoPEGylated FSH. Rats were subcutaneously injected with human chorionic gonadotropin and varying amounts of FSH for three days, and the average ovarian weight of the treatment group determined on day 4. rFSH-SA-PEG refers to recombinant FSH that has been glycoPEGylated with PEG (1 kDa). rFSH refers to non-glycoPEGylated FSH. Each treatment group contains 10 rats.

Figure 172, comprising Figures 172A and 172B, depicts the chromatogram of INF-β elution from a Superdex-75 column. Figure 172A depicts the entire chromatogram. Figure 172B depicts the boxed area of Figure 172A containing peaks 4 and 5 in greater detail.

Figure 173, comprising Figures 173A and 173B, depict MALDI analysis of glyeans enzymatically released from INF-β. Figure 173A depicts the MALDI analysis glycans released from native INF-β. Figure 173B depicts the MALDI analysis of glycans released from desialylated INF-β. The structures of the glycans have been determined by comparison of the peak spectrum with that of standard glycans. The glycan structures are depicted beside the peaks. Squares represent GlcNAc, triangles represent fucose, circles represent mannose, diamonds represent galactose and stars represent sialic acid.

Figure 174 depicts the lectin blot analysis of the sialylation of the desialylated INF-β. The blot on the right side is detected with *Maackia amurensis* agglutinin (MAA) labeled with digoxogenin (DIG) (Roche Applied Science, Indianapolis, IL) to detect α2,3-sialylation. The blot on the left is detected with *Erthrina cristagalli* lectin (ECL) labeled with biotin (Vector Laboratories, Burlingame, CA) to detect exposed galactose residues.

Figure 175 depicts the SDS-PAGE analysis of the products of the PEG (10 kDa) PEGylation reaction of INF-β. "-PEG" refers to INF-β before the PEGylation reaction. "+PEG" refers to INF-β after the PEGylation reaction.

Figure 176 depicts the SDS-PAGE analysis of the products of the PEG (20 kDa) PEGylation reaction of INF-β. "Unmodified" refers to INF-β before the PEGylation reaction. "Pegylated" refers to INF-β after the PEGylation reaction.

Figure 177 depicts the chromatogram of PEG (10 kDa) PEGylated INF-β elution from a Superdex-200 column.

Figure 178 depicts the results of a bioassay of peak fractions of PEG (10 kDa) PEGylated INF-β shown in the chromatogram depicted Figure INF-PEG 6.

Figure 179 depicts the chromatogram of PEG (20 kDa) PEGylated INF-β elution from a Superdex-200 column.

Figure 180, comprising Figures 180A and 180B, is two graphs depicting the MALDI-TOF spectrum of RNaseB (Figure 180A) and the HPLC profile of the oligosaccharides cleaved from RNaseB by N-Glycanase (Figure 180B). The majority of N-glycosylation sites of the peptide are modified with high mannose oligosaccharides consisting of 5 to 9 mannose residues.

Figure 181 is a scheme depicting the conversion of high mannose N-Glycans to hybrid N-Glycans. Enzyme 1 is α1,2-mannosidase, from *Trichodoma reesei* or *Aspergillus saitoi*. Enzyme 2 is GnT-I (β-1,2-*N*-acetyl glucosaminyl transferase I). Enzyme 3 is GalT-I (β1,4-galactosyltransfease 1). Enzyme 4 is α2,3-sialyltransferase or α2,6-sialyltransferase.

Figure 182, comprising Figures 182A and 182B, is two graphs depicting the MALDI-TOF spectrum of RNaseB treated with a recombinant *T. reesei* α1,2-mannosidase (Figure 182A) and the HPLC profile of the oligosaccharides cleaved by N-Glycanase from the modified RNaseB (Figure 182B).

Figure 183 is a graph depicting the MALDI-TOF spectrum of RNaseB treated with a commercially available α1,2-mannosidase purified from *A. saitoi* (Glyko & CalBioChem).

Figure 184 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 182 with a recombinant GnT-I (GlcNAc transferase-I).

Figure 185 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 184 with a recombinant GalT 1 (galactosyltransferase 1).

Figure 186 is a graph depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 185 with a recombinant ST3Gal III (α2,3-sialyltransferase III) using CMP-SA as the donor for the transferase.

Figure 187, comprising Figures 187A and 187B, are graphs depicting the MALDI-TOF spectrum of modified RNaseB by treating the product shown in Figure 185 with a recombinant ST3Gal III (α2,3-sialyltransferase III) using CMP-SA-PEG (10 kDa) as the donor for the transferase.

Figure 188 is a series of schemes depicting the conversion of high mannose N-glycans to complex N-glycans. Enzyme 1 is α1,2-mannosidase from *Trichoderma reesei* or *Aspergillus saitoi*. Enzyme 2 is GnT-I. Enzyme 3 is GalT 1. Enzyme 4 is α2,3-sialyltransferase or α2,6-sialyltransferase. Enzyme 5 is α-mannosidase II. Enzyme 6 is α-mannosidase. Enzyme 7 is GnT-II. Enzyme 8 is α1,6-mannosidase. Enzyme 9 is α1,3-mannosidase.

Figure 189 is a diagram of the linkage catalyzed by *N*-acetylglucosaminyltransferase I to VI (GnT I-VI). R = GlcNAcβ1,4GlcNAc-Asn-X.

Figure 190 is an image of an SDS-PAGE gel: standard (Lane 1); native transferrin (Lane 2); asialotransferrin (Lane 3); asialotransferrin and CMP-SA (Lane 4); Lanes 5 and 6, asialotransferrin and CMP-SA-PEG (1 kDa) at 0.5 mM and 5 mM, respectively; Lanes 7 and 8, asialotransferrin and CMP-SA-PEG (5 kDa) at 0.5 mM and 5 mM, respectively; Lanes 9 and 10, asialotransferrin and CMP-SA-PEG (10 kDa) at 0.5 mM and 5 mM, respectively.

Figure 191 is an image of an IEF gel: native transferrin (Lane 1); asialotransferrin (Lane 2); asialotransferrin and CMP-SA, 24 hr (Lane 3); asialotransferrin and CMP-SA, 96 hr (Lane 4) Lanes 5 and 6, asialotransferrin and CMP-SA-PEG (1 kDa) at 24 hr and 96 hr, respectively; Lanes 7 and 8, asialotransferrin and CMP-SA-PEG (5 kDa) at 24 hr and 96 hr, respectively; Lanes 9 and 10, asialotransferrin and CMP-SA-PEG (10 kDa) at 24 hr and 96 hr, respectively.

Figure 192 is a graph depicting the effects of EPO-SA-PEG (10 kDa), EPO-SA-PEG (20 kDa), and NESP on hemoglobin response in Sprague Dawley rats. Rats were dosed (indicated by arrows) by subcutaneous injection of 50 μg protein per Kg body weight. Values plotted are mean (n=5/group) increment in total blood hemoglobin baseline corrected against values obtained for control animals injected with vehicle alone.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods and compositions for the cell free *in vitro* addition and/or deletion of sugars to or from a peptide molecule in such a manner as to provide a glycopeptide molecule having a specific customized or desired glycosylation pattern, wherein the glycopeptide is produced at an industrial scale. In a preferred embodiment of the invention, the glycopeptide so produced has attached thereto a modified sugar that has been added to the peptide via an enzymatic reaction. A key feature of the invention is to take a peptide produced by any cell type and generate a core glycan structure on the peptide, following which the glycan structure is then remodeled *in vitro* to generate a glycopeptide having a glycosylation pattern suitable for therapeutic use in a mammal. More specifically, it is possible according to the present invention, to prepare a glycopeptide molecule having a modified sugar molecule or other compound conjugated thereto, such that

the conjugated molecule confers a beneficial property on the peptide. According to the present invention, the conjugate molecule is added to the peptide enzymatically because enzyme-based addition of conjugate molecules to peptides has the advantage of regioselectivity and stereoselectivity. The glycoconjugate may be added to the glycan on a peptide before or after glycosylation has been completed. In other words, the order of glycosylation with respect to glycoconjugation may be varied as described elsewhere herein. It is therefore possible, using the methods and compositions provided herein, to remodel a peptide to confer upon the peptide a desired glycan structure preferably having a modified sugar attached thereto. It is also possible, using the methods and compositions of the invention to generate peptide molecules having desired and or modified glycan structures at an industrial scale, thereby, for the first time, providing the art with a practical solution for the efficient production of improved therapeutic peptides.

Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (e.g., Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), which are provided throughout this document. The nomenclature used herein and the laboratory procedures used in analytical chemistry and organic syntheses described below are those well known and commonly employed in the art. Standard techniques or modifications thereof, are used for chemical syntheses and chemical analyses.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact

immunoglobulins derived from natural sources or from recombinant sources and can be immunoreaetive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

As used herein, the structure "  ", is the point of connection between an amino acid or an amino acid sidechain in the peptide chain and the glycan structure.

"N-linked" oligosaccharides are those oligosaccharides that are linked to a peptide backbone through asparagine, by way of an asparagine-N-acetylglucosamine linkage. N-linked oligosaccharides are also called "N-glycans." All N-linked oligosaccharides have a common pentasaccharide core of $Man_3GlcNAc_2$. They differ in the presence of, and in the number of branches (also called antennae) of peripheral sugars such as N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose and sialic acid. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

An "elemental trimannosyl core structure" refers to a glycan moiety comprising solely a trimannosyl core structure, with no additional sugars attached thereto. When the term

"elemental" is not included in the description of the "trimannosyl core structure," then the glycan comprises the trimannosyl core structure with additional sugars attached thereto. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

The term "elemental trimannosyl core glycopeptide" is used herein to refer to a glycopeptide having glycan structures comprised primarily of an elemental trimannosyl core structure. Optionally, this structure may also contain a core fucose molecule and/or a xylose molecule.

"O-linked" oligosaccharides are those oligosaccharides that are linked to a peptide backbone through threonine, serine, hydroxyproline, tyrosine, or other hydroxy-containing amino acids.

All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (i.e., Gal), followed by the configuration of the glycosidic bond (α or β), the ring bond (1 or 2), the ring position of the reducing saccharide involved in the bond (2, 3, 4, 6 or 8), and then the name or abbreviation of the reducing saccharide (*i.e.*, GlcNAc). Each saccharide is preferably a pyranose. For a review of standard glycobiology nomenclature see, Essentials of Glycobiology Varki et al. eds., 1999, CSHL Press.

The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* **2**: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

A peptide having "desired glycosylation", as used herein, is a peptide that comprises one or more oligosaccharide molecules which are required for efficient biological activity of the peptide.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

The "area under the curve" or "AUC", as used herein in the context of administering a peptide drug to a patient, is defined as total area under the curve that describes the concentration of drug in systemic circulation in the patient as a function of time from zero to infinity.

The term "half-life" or "t ½", as used herein in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most part, confined to the bloodstream, there can be at least two clearance half-lives. For some glycosylated peptides, rapid beta phase clearance may be mediated via receptors on macrophages, or endothelial cells that recognize terminal galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose, or fucose. Slower beta phase clearance may occur via renal glomerular filtration for molecules with an effective radius < 2 nm (approximately 68 kD) and/or specific or non-specific uptake and metabolism in tissues. GlycoPEGylation may cap terminal sugars (e.g. galactose or N-acetylgalactosamine) and thereby block rapid alpha phase clearance via receptors that recognize these sugars. It may also confer a larger effective radius and thereby decrease the volume of distribution and tissue uptake, thereby prolonging the late beta phase. Thus, the precise impact of glycoPEGylation on alpha phase and beta phase half-lives will vary depending upon the size, state of glycosylation, and other parameters, as is well known in the art. Further explanation of "half-life" is found in Pharmaceutical Biotechnology (1997, DFA Crommelin and RD Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 – 120).

The term "residence time", as used herein in the context of administering a peptide drug to a patient, is defined as the average time that drug stays in the body of the patient after dosing.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid nucleic acid encoding additional peptide sequence.

A "polynucleotide" means a single strand or parallel and anti-parallel strands of a nucleic acid. Thus, a polynucleotide may be either a single-stranded or a double-stranded nucleic acid.

The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides.

Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand which are located 5' to a reference point on the DNA are referred to as "upstream sequences"; sequences on the DNA strand which are 3' to a reference point on the DNA are referred to as "downstream sequences."

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of

other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a nucleic acid sequence encodes a protein if transcription and translation of mRNA corresponding to that nucleic acid produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that nucleic acid or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two peptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGGC share 50% homology.

As used herein, "homology" is used synonymously with "identity."

The determination of percent identity between two nucleotide or amino acid sequences can be accomplished using a mathematical algorithm. For example, a mathematical algorithm useful for comparing two sequences is the algorithm of Karlin and Altschul (1990, Proc. Natl. Acad. Sci. USA 87:2264-2268), modified as in Karlin and Altschul (1993, Proe. Natl. Acad. Sci. USA 90:5873-5877). This algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990, J. Mol. Biol. 215:403-410), and can be accessed, for example at the National Center for Biotechnology Information

(NCBI) world wide web site having the universal resource locator "http://www.ncbi.nlm.nih.gov/BLAST/". BLAST nucleotide searches can be performed with the NBLAST program (designated "blastn" at the NCBI web site), using the following parameters: gap penalty = 5; gap extension penalty = 2; mismatch penalty = 3; match reward = 1; expectation value 10.0; and word size = 11 to obtain nucleotide sequences homologous to a nueleic acid described herein. BLAST protein searches can be performed with the XBLAST program (designated "blastn" at the NCBI web site) or the NCBI "blastp" program, using the following parameters: expectation value 10.0, BLOSUM62 scoring matrix to obtain amino acid sequences homologous to a protein molecule described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997, Nucleic Acids Res. 25:3389-3402). Alternatively, PSI-Blast or PHI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.) and relationships between molecules which share a common pattern. When utilizing BLAST, Gapped BLAST, PSI-Blast, and PHI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

A "heterologous nucleic acid expression unit" encoding a peptide is defined as a nucleic acid having a coding sequence for a peptide of interest operably linked to one or more expression control sequences such as promoters and/or repressor sequences wherein at least one of the sequences is heterologous, i. e., not normally found in the host cell.

By describing two polynucleotides as "operably linked" is meant that a single-stranded or double-stranded nucleic acid moiety comprises the two polynucleotides arranged within the nucleic acid moiety in such a manner that at least one of the two polynucleotides is able to exert a physiological effect by which it is characterized upon the other. By way of example, a promoter operably linked to the coding region of a nucleic acid is able to promote transcription of the coding region.

As used herein, the term "promoter/regulatory sequence" means a nucleic aeid sequence which is required for expression of a gene product operably linked to the

promoter/regulator sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive promoter is a promoter which drives expression of a gene to which it is operably linked, in a constant manner in a cell. By way of example, promoters which drive expression of cellular housekeeping genes are considered to be constitutive promoters.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequenees operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids

(e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

A "genetically engineered" or "recombinant" cell is a cell having one or more modifications to the genetic material of the cell. Such modifications are seen to include, but are not limited to, insertions of genetic material, deletions of genetic material and insertion of genetic material that is extrachromasomal whether such material is stably maintained or not.

A "peptide" is an oligopeptide, polypeptide, peptide, protein or glycoprotein. The use of the term "peptide" herein includes a peptide having a sugar molecule attached thereto when a sugar molecule is attached thereto.

As used herein, "native form" means the form of the peptide when produced by the cells and/or organisms in which it is found in nature. When the peptide is produced by a plurality of cells and/or organisms, the peptide may have a variety of native forms.

"Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a peptide. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine and homoarginine are also included. Amino acids that are not nucleic acid-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L – isomer thereof. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see,* Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with a modified sugar as set forth herein.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-

carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is linked to a hydrogen, a earboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

As used herein, amino acids are represented by the full name thereof, by the three letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following Table 1:

Table 1. Amino acids, and the three letter and one letter codes.

Full Name	Three-Letter Code	One-Letter Code
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Tyrosine	Tyr	Y
Cysteine	Cys	C
Asparagine	Asn	N
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W

The present invention also provides for analogs of proteins or peptides which comprise a protein as identified above. Analogs may differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do

not affect sequence, or by both. For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its funetion. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro*, chemical derivatization of peptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a peptide during its synthesis and processing or in further processing steps; e.g., by exposing the peptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

It will be appreciated, of course, that the peptides may incorporate amino acid residues which are modified without affecting activity. For example, the termini may be derivatized to include blocking groups, i.e. chemical substituents suitable to protect and/or stabilize the N- and C-termini from "undesirable degradation", a term meant to encompass any type of enzymatic, chemical or biochemical breakdown of the compound at its termini which is likely to affect the function of the compound, i.e. sequential degradation of the compound at a terminal end thereof.

Blocking groups include protecting groups conventionally used in the art of peptide chemistry which will not adversely affect the *in vivo* activities of the peptide. For example, suitable N-terminal blocking groups can be introduced by alkylation or acylation of the N-terminus. Examples of suitable N-terminal blocking groups include C_1-C_5 branched or unbranched alkyl groups, acyl groups such as formyl and acetyl groups, as well as substituted

forms thereof, such as the acetamidomethyl (Acm), Fmoc or Boc groups. Desamino analogs of amino acids are also useful N-terminal blocking groups, and can either be coupled to the N-terminus of the peptide or used in place of the N-terminal reside. Suitable C-terminal blocking groups, in which the carboxyl group of the C-terminus is either incorporated or not, include esters, ketones or amides. Ester or ketone-forming alkyl groups, particularly lower alkyl groups such as methyl, ethyl and propyl, and amide-forming amino groups such as primary amines (-NH_2), and mono- and di-alkylamino groups such as methylamino, ethylamino, dimethylamino, diethylamino, methylethylamino and the like are examples of C-terminal blocking groups. Descarboxylated amino acid analogues such as agmatine are also useful C-terminal blocking groups and can be either coupled to the peptide's C-terminal residue or used in place of it. Further, it will be appreciated that the free amino and carboxyl groups at the termini can be removed altogether from the peptide to yield desamino and descarboxylated forms thereof without affect on peptide activity.

Other modifications can also be incorporated without adversely affecting the activity and these include, but are not limited to, substitution of one or more of the amino acids in the natural L-isomeric form with amino acids in the D-isomeric form. Thus, the peptide may include one or more D-amino acid resides, or may comprise amino acids which are all in the D-form. Retro-inverso forms of peptides in accordance with the present invention are also contemplated, for example, inverted peptides in which all amino acids are substituted with D-amino acid forms.

Acid addition salts of the present invention are also contemplated as functional equivalents. Thus, a peptide in accordance with the present invention treated with an inorganic acid such as hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, and the like, or an organic acid such as an acetic, propionic, glycolic, pyruvic, oxalic, malic, malonic, succinic, maleic, fumaric, tataric, citric, benzoic, cinnamic, mandelic, methanesulfonic, ethanesulfonic, p-toluenesulfonic, salicyclic and the like, to provide a water soluble salt of the peptide is suitable for use in the invention.

Also included are peptides which have been modified using ordinary moleeular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such peptides include those containing residues other than naturally occurring L-amino

acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

As used herein, the term "MALDI" is an abbreviation for Matrix Assisted Laser Desorption Ionization. During ionization, SA-PEG (sialic acid-poly(ethylene glycol)) can be partially eliminated from the N-glycan structure of the glycoprotein.

As used herein, the term "glycosyltransferase," refers to any enzyme/protein that has the ability to transfer a donor sugar to an acceptor moiety.

As used herein, the term "modified sugar," refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (*e.g.*, glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides.

The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers, therapeutic moieties, diagnostic moieties, biomolecules and the like. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences or be composed of a single amino acid, e.g. poly(lysine). Similarly, saccharides can be of mixed sequence or composed of a single saccharide subunit, e.g., dextran, amylose, chitosan, and poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol). Poly(ethylene imine) is an exemplary polyamine, and poly(aspartic) acid is a representative poly(carboxylic acid).

"Poly(alkylene oxide)" refers to a genus of compounds having a polyether backbone. Poly(alkylene oxide) species of use in the present invention include, for example, straight- and branched-chain species. Moreover , exemplary poly(alkylene oxide) species can terminate in one or more reactive, activatable, or inert groups. For example, poly(ethylene

glycol) is a poly(alkylene oxide) consisting of repeating ethylene oxide subunits, which may or may not include additional reactive, activatable or inert moieties at either terminus. Useful poly(alkylene oxide) species include those in which one terminus is "capped" by an inert group, e.g., monomethoxy-poly(alkylene oxide). When the molecule is a branched species, it may include multiple reactive, activatable or inert groups at the termini of the alkylene oxide chains and the reactive groups may be either the same or different. Derivatives of straight-chain poly(alkylene oxide) species that are heterobifunctional are also known in the art.

The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which an agent (e.g., water-soluble polymer, therapeutic moiety, biomolecule) is covalently attached. In the methods of the invention, the "glycosyl linking group" becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A "glycosyl linking group" is generally derived from a "modified sugar" by the enzymatic attachment of the "modified sugar" to an amino acid and/or glycosyl residue of the peptide. More specifically, a "glycosyl linking group," as used herein, refers to a moiety that covalently joins a "modifying group," as discussed herein, and an amino acid residue of a peptide. The glycosyl linking group-modifying group adduct has a structure that is a substrate for an enzyme. The enzymes for which the glycosyl linking group-modifying group adduct are substrates are generally those capable of transferring a saccharyl moiety onto an amino acid residue of a peptide, e.g, a glycosyltransferase, amidase, glycosidase, trans-sialidase, etc. The "glycosyl linking group" is interposed between, and covalently joins a "modifying group" and an amino acid residue of a peptide.

An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the individual saccharide monomer that links the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure. An exemplary "intact glycosyl linking group" includes at least one intact, e.g., non-degraded, saccharyl moiety that is covalently attached to an amino acid residue on a peptide. The remainder of the "linking group" can have substantially any structure. For example, the modifying group is optionally linked directly to the intact saccharyl moiety. Alternatively,

the modifying group is linked to the intact saccharyl moiety via a linker arm. The linker arm can have substantially any structure determined to be useful in the selected embodiment. In an exemplary embodiment, the linker arm is one or more intact saccharyl moieties, i.e. "the intact glycosyl linking group" resembles an oligosaccharide. Another exemplary intact glycosyl linking group is one in which a saccharyl moiety attached, directly or indireetly, to the intact saccharyl moiety is degraded and derivatized (e.g., periodate oxidation followed by reductive amination). Still a further linker arm includes the modifying group attached to the intact saccharyl moiety, directly or indirectly, via a cross-linker, such as those described herein or analogues thereof.

"Degradation," as used herein refers to the removal of one or more carbon atoms from a saccharyl moiety.

The terms "targeting moiety" and "targeting agent", as used herein, refer to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art.

As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is linked to a carrier, e.g., multivalent agents. Therapeutic moiety also includes peptides, and constructs that include peptides. Exemplary peptides include those disclosed in Figure 28 and Tables 6 and 7, herein. "Therapeutic moiety" thus means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is linked to a carrier, e.g., multivalent agents.

As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents,

anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, *e.g.* TNF-α. Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF-α.

As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diphtheria toxin, and snake venom (e.g., cobra venom).

As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60 and technetium. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g. EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc*). *See*, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge, 1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for

example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al., Bioconjugate Chem.*, **9:** 108-117 (1998); Song *et al., Bioconjugate Chem.*, **8:** 249-255 (1997).

As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the activity of the conjugate activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, intrathecal administration, or the implantation of a slow-release device *e.g.*, a mini-osmotic pump, to the subject.

The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about

92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

"Commercial scale" as used herein means about one or more gram of final product produced in the method.

"Essentially each member of the population," as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. "Essentially each member of the population" speaks to the "homogeneity" of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more prefcrably at least about 95% homogenous.

"Homogeneity," refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, e.g., liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF), capillary electrophoresis, and the like.

"Substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that

are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). For example, in the case of a α1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Galβ1,4-GlcNAc-R and sialylated analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (*e.g.*, fucosylated Galβ1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

Description of the Invention

I. Method to Remodel Glycan Chains

The present invention includes methods and compositions for the *in vitro* addition and/or deletion of sugars to or from a glycopeptide molecule in such a manner as to provide a peptide molecule having a specific customized or desired glycosylation pattern, preferably including the addition of a modified sugar thereto. A key feature of the invention therefore is to take a peptide produced by any cell type and generate a core glycan structure on the peptide, following which the glycan structure is then remodeled *in vitro* to generate a peptide having a glycosylation pattern suitable for therapeutic use in a mammal.

The importance of the glycosylation pattern of a peptide is well known in the art as are the limitations of present *in vivo* methods for the production of properly glycosylated peptides, particularly when these peptides are produced using recombinant DNA methodology. Moreover, until the present invention, it has not been possible to generate glycopeptides having a desired glycan structure thereon, wherein the peptide can be produced at industrial scale.

In the present invention, a peptide produced by a cell is enzymatically treated *in vitro* by the systematic addition of the appropriate enzymes and substrates therefor, such that sugar

moieties that should not be present on the peptide are removed, and sugar moieties, optionally including modified sugars, that should be added to the peptide are added in a manner to provide a glycopeptide having "desired glycosylation", as defined elsewhere herein.

A. Method to remodel N-linked glycans

In one aspect, the present invention takes advantage of the fact that most peptides of commercial or pharmaceutical interest comprise a common five sugar structure referred to herein as the trimannosyl core, which is N-linked to asparagine at the sequence Asn-X-Ser/Thr on a peptide chain. The elemental trimannosyl core consists essentially of two N-acetylglucosamine (GlcNAc) residues and three mannose (Man) residues attached to a peptide, i.e., it comprises these five sugar residues and no additional sugars, except that it may optionally include a fucose residue. The first GlcNAc is attached to the amide group of the asparagine and the second GlcNAc is attached to the first via a β1,4 linkage. A mannose residue is attached to the second GlcNAc via a β1,4 linkage and two mannose residues are attached to this mannose via an α1,3 and an α1,6 linkage respectively. A schematic depiction of a trimannosyl core structure is shown in Figure 1, left side. While it is the case that glycan structures on most peptides comprise other sugars in addition to the trimannosyl core, the trimannosyl core structure represents an essential feature of N-linked glycans on mammalian peptides.

The present invention includes the generation of a peptide having a trimannosyl core structure as a fundamental element of the structure of the glycan molecules contained thereon. Given the variety of cellular systems used to produce peptides, whether the systems are themselves naturally occurring or whether they involve recombinant DNA methodology, the present invention provides methods whereby a glycan molecule on a peptide produced in any cell type can be reduced to an elemental trimannosyl core structure. Once the elemental trimannosyl core structure has been generated then it is possible using the methods described herein, to generate *in vitro*, a desired glycan structure on the peptide which confers on the peptide one or more properties that enhances the therapeutic effectiveness of the peptide.

It should be clear from the discussion herein that the term "trimannosyl core" is used to describe the glycan structure shown in Figure 1, left side. Glycopeptides having a trimannosyl core structure may also have additional sugars added thereto, and for the most part, do have additional structures added thereto irrespective of whether the sugars give rise

to a peptide having a desired glycan structure. The term "elemental trimannosyl core structure" is defined elsewhere herein. When the term "elemental" is not included in the description of the "trimannosyl core structure," then the glycan comprises the trimannosyl core structure with additional sugars attached to the mannose sugars.

The term "elemental trimannosyl core glycopeptide" is used herein to refer to a glycopeptide having glycan structures comprised primarily of an elemental trimannosyl core structure. However, it may also optionally contain a fucose residue attached thereto. As discussed herein, elemental trimannosyl core glycopeptides are one optimal, and therefore preferred, starting material for the glycan remodeling processes of the invention.

Another optimal starting material for the glycan remodeling process of the invention is a glycan structure having a trimannosyl core wherein one or two additional GlcNAc residues are added to each of the α1,3 and the α1,6 mannose residues (see for example, the structure on the second line of Figure 2, second structure in from the left of the figure). This structure is referred to herein as "Man3GlcNAc4." When the structure is monoantenary, the structure is referred to herein as "Man3GlcNAc3." Optionally, this structure may also contain a core fucose molecule. Once the Man3GlcNAc3 or Man3GlcNAc4 structure has been generated then it is possible using the methods described herein, to generate *in vitro*, a desired glycan structure on the glycopeptide which confers on the glycopeptide one or more properties that enhances the therapeutic effectiveness of the peptide.

In their native form, the N-linked glycopeptides of the invention, and particularly the mammalian and human glycopeptides useful in the present invention, are N-linked glycosylated with a trimannosyl core structure and one or more sugars attached thereto.

The terms "glycopeptide" and "glycopolypeptide" are used synonymously herein to refer to peptide chains having sugar moieties attached thereto. No distinction is made herein to differentiate small glycopolypeptides or glycopeptides from large glycopolypeptides or glycopeptides. Thus, hormone molecules having very few amino acids in their peptide chain (e.g., often as few as three amino acids) and other much larger peptides are included in the general terms "glycopolypeptide" and "glycopeptide," provided they have sugar moieties attached thereto. However, the use of the term "peptide" does not preclude that peptide from being a glycopeptide.

An example of an N-linked glycopeptide having desired glycosylation is a peptide having an N-linked glycan having a trimannosyl core with at least one GlcNAc residue attached thereto. This residue is added to the trimannosyl core using N-acetyl glucosaminyltransferase I (GnT-I). If a second GlcNAc residue is added, N-acetyl glucosaminyltransferase II (GnT-II) is used. Optionally, additional GlcNAc residues may be added with GnT-IV and/or GnT-V, and a third bisecting GlcNAc residue may be attached to the β1,4 mannose of the trimannosyl core using N-acetyl glucosaminyltransferase III (GnT-III). Optionally, this structure may be extended by treatment with β1,4 galactosyltransferase to add a galactose residue to each non-bisecting GlcNAc, and even further optionally, using α2,3 or α2,6-sialyltransferase enzymes, sialic acid residues may be added to each galactose residue. The addition of a bisecting GlcNAc to the glycan is not required for the subsequent addition of galactose and sialic acid residues; however, with respect to the substrate affinity of the rat and human GnT-III enzymes, the presence of one or more of the galactose residues on the glycan precludes the addition of the bisecting GlcNAc in that the galactose-containing glycan is not a substrate for these forms of GnT-III. Thus, in instances where the presence of the bisecting GlcNAc is desired and these forms of GnT-III are used, it is important should the glycan contain added galactose and/or sialic residues, that they are removed prior to the addition of the bisecting GlcNAc. Other forms of GnT-III may not require this specific order of substrates for their activity. In the more preferred reaction, a mixture of GnT-I, GnT-II and GnT-III is added to the reaction mixture so that the GlcNAc residues can be added in any order.

Examples of glycan structures which represent the various aspects of peptides having "desired glycosylation" are shown in the drawings provided herein. The precise procedures for the *in vitro* generation of a peptide having "desired glycosylation" are described elsewhere herein. However, the invention should in no way be construed to be limited solely to any one glycan structure disclosed herein. Rather, the invention should be construed to include any and all glycan structures which can be made using the methodology provided herein.

In some cases, an elemental trimannosyl core alone may constitute the desired glycosylation of a peptide. For example, a peptide having only a trimannosyl core has been

shown to be a useful component of an enzyme employed to treat Gaucher disease (Mistry et al., 1966, Lancet 348: 1555-1559; Bijsterbosch et al., 1996, Eur. J. Bioehem. 237:344-349) .

According to the presnt invention, the following procedures for the generation of peptides having desired glycosylation become apparent.

a) Beginning with a glycopeptide having one or more glycan molecules which have as a common feature a trimannosyl core structure and at least one or more of a heterogeneous or homogeneous mixture of one or more sugars added thereto, it is possible to increase the proportion of glycopeptides having an elemental trimannosyl core structure as the sole glycan structure or which have Man3GlcNAc3 or Man3GlcNAc4 as the sole glycan structure. This is accomplished *in vitro* by the systematic addition to the glycopeptide of an appropriate number of enzymes in an appropriate sequence which cleave the heterogeneous or homogeneous mixture of sugars on the glycan structure until it is reduced to an elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structurc. Spccific examples of how this is accomplished will depend on a variety of factors including in large part the type of cell in which the peptide is produced and therefore the degree of complexity of the glycan structure(s) present on the peptide initially produced by the cell. Examples of how a complex glycan structure can be reduced to an elemental trimannosyl core or a Man3GlcNAc3 or Man3GlcNAc4 structure are presented in Figure 2 or are described in detail elsewhere herein.

b) It is possible to generate a peptide having an elemental trimannosyl core structure as the sole glycan structure on the peptide by isolating a naturally occurring cell whose glycosylation machinery produces such a peptide. DNA encoding a peptide of choice is then transfected into the cell wherein the DNA is transcribed, translated and glycosylated such that the peptide of choice has an elemental trimannosyl core structure as the sole glycan structure thereon. For example, a cell lacking a functional GnT-I enzyme will produce several types of glycopeptides. In some instances, these will bc glycopeptides having no additional sugars attached to the trimannosyl core. However, in other instances, the peptides produced may have two additional mannose residues attached to the trimannosyl core, resulting in a Man5 glycan. This is also a desired starting material for the remodeling process of the present invention. Specific examples of the generation of such glycan structures are described herein.

c) Alternatively, it is possible to genetically engineer a cell to confer upon it a specific glycosylation machinery such that a peptide having an elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structure as the sole glycan structure on the peptide is produced. DNA encoding a peptide of choice is then transfected into the cell wherein the DNA is transcribed, translated and glycosylated such that the peptide of choice has an increased number of glycans comprising solely an elemental trimannosyl core structure. For example, certain types of cells that are genetically engineered to lack GnT-I, may produce a glycan having an elemental trimannosyl core structure, or, depending on the cell, may produce a glycan having a trimannosyl core plus two additional mannose residues attached thereto (Man5). When the cell produces a Man5 glycan structure, the cell may be further genetically engineered to express mannosidase 3 which cleaves off the two additional mannose residues to generate the trimannosyl core. Alternatively, the Man5 glycan may be incubated *in vitro* with mannosidase 3 to have the same effect.

d) When a peptide is expressed in an insect cell, the glycan on the peptide comprises a partially complex chain. Insect cells also express hexosaminidase in the cells which trims the partially complex chain back to a trimannosyl core structure which can then be remodeled as described herein.

e) It is readily apparent from the discussion in b), c) and d) that it is not necessary that the cells produce only peptides having elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structures attached thereto. Rather, unless the cells described in b) and c) produce peptides having 100% elemental trimannosyl core structures (i.e., having no additional sugars attached thereto) or 100% of Man3GlcNAc3 or Man3GlcNAc4 structures, the cells in fact produce a heterogeneous mixture of peptides having, in combination, elemental trimannosyl core structures, or Man3GlcNAc3 or Man3GlcNAc4 structures, as the sole glycan structure in addition to these structures having additional sugars attached thereto. The proportion of peptides having a trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 structures having additional sugars attached thereto, as opposed to those having one structure, will vary depending on the cell which produces them. The complexity of the glycans (i.e. which and how many sugars are attached to the trimannosyl core) will also vary depending on the cell which produces them.

f) Once a glycopeptide having an elemental trimannosyl core or a trimannosyl core with one or two GlcNAc residues attached thereto is produced by following a), b) or c) above, according to the present invention, additional sugar molecules are added *in vitro* to the trimannosyl core structure to generate a peptide having desired glycosylation (i.e., a peptide having an *in vitro* customized glycan structure).

g) However, when it is the case that a peptide having an elemental trimannosyl core or Man3GlcNAc4 structure with some but not all of the desired sugars attached thereto is produced, then it is only necessary to add any remaining desired sugars without reducing the glycan structure to the elemental trimannosyl core or Man3GlcNAc4 structure. Therefore, in some cases, a peptide having a glycan structure having a trimannosyl core structure with additional sugars attached thereto, will be a suitable substrate for remodeling.

Isolation of an elemental trimannosyl core glycopeptide

The elemental trimannosyl core or Man3GlcNAc3 or Man3GlcNAc4 glycopeptides of the invention may be isolated and purified, if necessary, using techniques well known in the art of peptide purification. Suitable techniques include chromatographic techniques, isoelectric focusing techniques, ultrafiltration techniques and the like. Using any such techniques, a composition of the invention can be prepared in which the glycopeptides of the invention are isolated from other peptides and from other components normally found within cell culture media. The degree of purification can be, for example, 90% with respect to other peptides or 95%, or even higher, e.g., 98%. See, e.g., Deutscher et al. (ed., 1990, Guide to Protein Purification, Harcourt Brace Jovanovich, San Diego).

The heterogeneity of N-linked glycans present in the glycopeptides produced by the prior art methodology generally only permits the isolation of a small portion of the target glycopeptides which can be modified to produce desired glycopeptides. In the present methods, large quantities of elemental trimannosyl core glycopeptides and other desired glycopeptides, including Man3GlcNAc3 or Man3GlcNAc4 glycans, can be produced which can then be further modified to generate large quantities of peptides having desired glycosylation.

Specific enrichment of any particular type of glycan linked to a peptide may be accomplished using lectins which have an affinity for the desired glycan. Such techniques are well known in the art of glycobiology.

A key feature of the invention which is described in more detail below, is that onee a core glycan structure is generated on any peptide, the glycan structure is then remodeled *in vitro* to generate a peptide having desired glycosylation that has improved therapeutic use in a mammal. The mammal may be any type of suitable mammal, and is preferably a human.

The various scenarios and the precise methods and compositions for generating peptides with desired glycosylation will become evident from the disclosure which follows.

The ultimate objective of the production of peptides for therapeutic use in mammals is that the peptides should comprise glycan structures that facilitate rather than negate the therapeutic benefit of the peptide. As disclosed throughout the present specification, peptides produced in cells may be treated *in vitro* with a variety of enzymes which catalyze the cleavage of sugars that should not be present on the glycan and the addition of sugars which should be present on the glycan such that a peptide having desired glycosylation and thus suitable for therapeutic use in mammals is generated. The generation of different glycoforms of peptides in cells is described above. A variety of mechanisms for the generation of peptides having desired glycosylation is now described, where the starting material i.e., the peptide produced by a cell may differ from one cell type to another. As will become apparent from the present disclosure, it is not necessary that the starting material be uniform with respect to its glycan composition. However, it is preferable that the starting material be enriched for certain glycoforms in order that large quantities of end product, i.e., correctly glycosylated peptides are produced.

In a preferred embodiment according to the present invention, the degradation and synthesis events that result in a peptide having desired glycosylation involve at some point, the generation of an elemental trimannosyl core structure or a Man3GlcNAc3 or Man3GlcNAc4 structure on the peptide.

The present invention also provides means of adding one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not

present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a peptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the peptide. See for example WO 98/31826.

Addition or removal of any carbohydrate moieties present on the peptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the peptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin et al., 1987, Arch. Biochem. Biophys. 259: 52 and by Edge et al., 1981, Anal. Biochem. 118: 131. Enzymatic cleavage of carbohydrate moieties on peptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., 1987, Meth. Enzymol. 138: 350.

Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) sites for N- and O-glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Dealing specifically with the examples shown in several of the figures provided herein, a description of the sequence of *in vitro* enzymatic reactions for the production of desired glycan structures on peptides is now presented. The precise reaction conditions for

each of the enzymatic conversions disclosed below are well known to those skilled in the art of glycobiology and are therefore not repeated here. For a review of the reaction conditions for these types of reactions, see Sadler et al., 1982, Methods in Enzymology 83:458-514 and references cited therein.

In Figure 1 there is shown the structure of an elemental trimannosyl core glycan on the left side. It is possible to convert this structure to a complete glycan structure having a bisecting GlcNAc by incubating the elemental trimannosyl core structure in the presence of GnT-I, followed by GnT-II, and further followed by GnT-III, and a sugar donor comprising UDP-GlcNAc, wherein GlcNAc is sequentially added to the elemental trimannosyl core structure to generate a trimannosyl core having a bisecting GlcNAc. In some instances, for example when remodeling Fc glycans as described herein, the order of addition of GnT-I, GnT-II and GnT-III may be contrary to that reported in the literature. The bisecting GlcNAc structure may be produced by adding a mixture of GnT-I, GnT-II and GnT-III and UDP-GlcNAc to the reaction mixture

In Figure 3 there is shown the conversion of a bisecting GlcNAc containing trimannosyl core glycan to a complex glycan structure comprising galactose and N-acetyl neuraminic acid. The bisecting GlcNAc containing trimannosyl core glycan is first incubated with galactosyltransferase and UDP-Gal as a donor molecule, wherein two galactose residues are added to the peripheral GlcNAc residues on the molecule. The enzyme NeuAc-transferase is then used to add two NeuAc residues one to each of the galactose residues.

In Figure 4 there is shown the conversion of a high mannose glycan structure to an elemental trimannosyl core glycan. The high mannose glycan (Man9) is incubated sequentially in the presence of the mannosidase 1 to generate a Man5 structure and then in the presence of mannosidase 3, wherein all but three mannose residues are removed from the glycan. Alternatively, incubation of the Man9 structure may be trimmed back to the trimannosyl core structure solely by incubation in the presence of mannosidase 3. According to the schemes presented in Figures 1 and 3 above, conversion of this elemental trimannosyl core glycan to a complex glycan molecule is then possible.

In Figure 5 there is shown a typical complex N-linked glycan structure produced in plant cells. It is important to note that when plant cells are deficient in GnT-I enzymatic activity, xylose and fucose cannot be added to the glycan. Thus, the use of GnT-I knock-out

cells provides a particular advantage in the present invention in that these cells produce peptides having an elemental trimannosyl core onto which additional sugars can be added without performing any "trimming back" reactions. Similarly, in instances where the structure produced in a plant cell may be of the Man5 variety of glycan, if GnT-I is absent in these cells, xylose and fucose cannot be added to the structure. In this case, the Man5 structure may be trimmed back to an elemental trimannosyl core (Man3) using mannosidase 3. According to the methods provided herein, it is now possible to add desired sugar moieties to the trimannosyl core to generate a desired glycan structure.

In Figure 6 there is shown a typical complex N-linked glycan structure produced in insect cells. As is evident, additional sugars, such as, for example, fucose may also be present. Further although not shown here, insect cells may produce high mannose glycans having as many as nine mannose residues and may have additional sugars attached thereto. It is also the case in insect cells that GnT-I knock out cells prevent the addition of fucose residues to the glycan. Thus, production of a peptide in insect cells may preferably be accomplished in a GnT-I knock out cell. The glycan thus produced may then be trimmed back *in vitro* if necessary using any of the methods and schemes described herein, and additional sugars may be added *in vitro* thereto also using the methods and schemes provided herein.

In Figure 2 there is shown glycan structures in various stages of completion. Specifically, the *in vitro* enzymatic generation of an elemental trimannosyl core structure from a complex carbohydrate glycan structure which does not contain a bisecting GlcNAc residue is shown. Also shown is the generation of a glycan structure therefrom which contains a bisecting GlcNAc. Several intermediate glycan structures which can be produced are shown. These structures can be produced by cells, or can be produced in the *in vitro* trimming back reactions described herein. Sugar moieties may be added *in vitro* to the elemental trimannosyl core structure, or to any suitable intermediate structure in order that a desired glycan is produced.

In Figure 7 there is shown a series of possible *in vitro* reactions which can be performed to trim back and add onto glycans beginning with a high mannose structure. For example, a Man9 glycan may be trimmed using mannosidase 1 to generate a Man5 glycan, or it may be trimmed to a trimannosyl core using mannosidase 3 or one or more mierobial

mannosidases. GnT-I and or GnT-II may then be used to transfer additional GlcNAc residues onto the glycan. Further, there is shown the situation which would not occur when the glycan molecule is produced in a cell that does not have GnT-I (see shaded box). For example, fucose and xylose may be added to a glycan only when GnT-I is active and facilitates the transfer of a GlcNAc to the molecule.

Figure 8 depicts well known strategies for the synthesis of biantennary, triantennary and even tetraantennary glycan structures beginning with the trimannosyl core structure. According to the methods of the invention, it is possible to synthesize each of these structures *in vitro* using the appropriate enzymes and reaction conditions well known in the art of glycobiology.

Figure 9 depicts two methods for synthesis of a monoantennary glycan structure beginning from a high mannose (6 to 9 mannose moieties) glycan structures. A terminal sialic acid-PEG moiety may be added in place of the sialic acid moiety in accordance with glycoPEGylation methodology described herein. In the first method, endo-H is used to cleave the glycan structure on the peptide back to the first GlcNAc residue. Galactose is then added using galactosyltransferase and sialylated-PEG is added as described elsewhere herein. In the second method, mannosidase I is used to cleave mannose residues from the glycan structure in the peptide. A galactose residue is added to one arm of the remaining mannose residues which were cleaved off the glycan using Jack Bean α-mannosidase. Sialylated-PEG is then added to this structure as directed.

Figure 10 depicts two additional methods for synthesis of a monoantennary glycan structures beginning from high mannose (6 to 9 mannose moieties) glycan structure. As in Figure 9, a terminal sialic acid-PEG moiety may be added in place of the sialic acid moiety in accordance with the glycoPEGylation methodology described herein. In the situation described here, some of the mannose residues from the arm to which sialylated-PEG is not added, are removed.

In Figure 11 there is shown a scheme for the synthesis of yet more complex carbohydrate structures beginning with a trimannosyl core structure. For example, a scheme for the *in vitro* production of Lewis x and Lewis a antigen structures, which may or may not be sialylated is shown. Such structures when present on a peptide may confer on the peptide immunological advantages for upregulating or downregulating the immune response. In

addition, such structures are useful for targeting the peptide to specific cells, in that these types of structures are involved in binding to cell adhesion peptides and the like.

Figure 12 is an exemplary scheme for preparing an array of O-linked peptides originating with serine or threonine.

Figure 13 is a series of diagrams depicting the four types of O-linked glycan structure termed cores 1 through 4. The core structure is outlined in dotted lines. Sugars which may also be included in this structure include sialic acid residues added to the galactose residues, and fucose residues added to the GlcNAc residues.

Thus, in preferred embodiments, the present invention provides a method of making an N-linked glycosylated glycopeptide by providing an isolated and purified glycopeptide to which is attached an elemental trimannosyl core or a Man3GlcNAc4 structure, contacting the glycopeptide with a glycosyltransferase enzyme and a donor molecule having a glycosyl moiety under conditions suitable to transfer the glycosyl moiety to the glycopeptide. Customization of a trimannosyl core glycopeptide or Man3GlcNAc4 glycopeptide to produce a peptide having a desired glycosylation pattern is then accomplished by the sequential addition of the desired sugar moieties, using techniques well known in the art.

Determination of Glycan Primary Structure

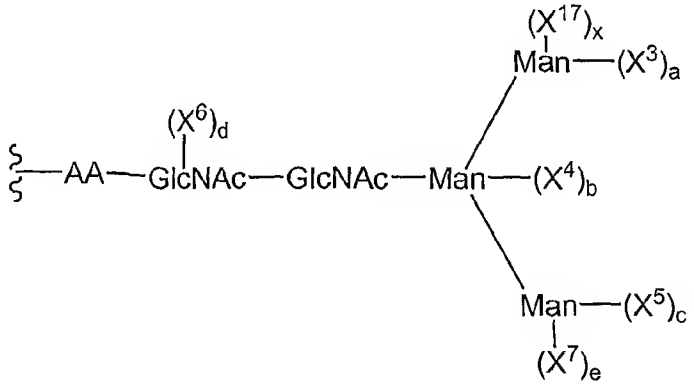
When an N-linked glycopeptide is produced by a cell, as noted elsewhere herein, it may comprise a heterogeneous mixture of glycan structures which must be reduced to a common, generally elemental trimannosyl core or Man3GlcNAc4 structure, prior to adding other sugar moieties thereto. In order to determine exactly which sugars should be removed from any particular glycan structure, it is sometimes necessary that the primary glycan structure be identified. Techniques for the determination of glycan primary structure are well know in the art and are described in detail, for example, in Montreuil, "Structure and Biosynthesis of Glycopeptides" In Polysaccharides in Medicinal Applications, pp. 273-327, 1996, Eds. Severian Damitriu, Marcel Dekker, NY. It is therefore a simple matter for one skilled in the art of glycobiology to isolate a population of peptides produced by a cell and determine the structure(s) of the glycans attached thereto. For example, efficient methods are available for (i) the splitting of glycosidic bonds either by chemical cleavage such as hydrolysis, acetolysis, hydrazinolysis, or by nitrous deamination; (ii) complete methylation followed by hydrolysis or methanolysis and by gas-liquid chromatography and mass

spectroscopy of the partially methylated monosaccharides; and (iii) the definition of anomeric linkages between monosaccharides using exoglycosidases, which also provide insight into the primary glycan structure by sequential degradation. In particular, the techniques of mass spectroscopy and nuclear magnetic resonance (NMR) spectrometry, especially high field NMR have been successfully used to determine glycan primary structure.

Kits and equipment for carbohydrate analysis are also commercially available. Fluorophore Assisted Carbohydrate Electrophoresis (FACE®) is available from Glyko, Inc. (Novato, CA). In FACE analysis, glycoconjugates are released from the peptide with either Endo H or N-glycanase (PNGase F) for N-linked glycans, or hydrazine for Ser/Thr linked glycans. The glycan is then labeled at the reducing end with a fluorophore in a non-structure discriminating manner. The fluorophore labeled glycans are then separated in polyacrylamide gels based on the charge/mass ratio of the saccharide as well as the hydrodynamic volume. Images are taken of the gel under UV light and the composition of the glycans are determined by the migration distance as compared with the standards. Oligosaccharides can be sequenced in this manner by analyzing migration shifts due to the sequential removal of saccharides by exoglycosidase digestion.

Exemplary embodiment

The remodeling of N-linked glycosylation is best illustrated with reference to Formula 1:



where X^3, X^4, X^5, X^6, X^7 and X^{17} are (independently selected) monosaccharide or oligosaccharide residues; and

a, b, c, d, e and x are (independently selected) 0, 1 or 2, with the proviso that at least one member selected from a, b, c, d, e and x are 1 or 2.

Formula 1 deseribes glycan structure comprising the tri-mannosyl core, which is preferably covalently linked to an asparagine residue on a peptide backbone. Preferred expression systems will express and secrete exogenous peptides with N-linked glycans comprising the tri-mannosyl core. Using the remodeling method of the invention, the glycan structures on these peptides can be conveniently remodeled to any glycan structure desired. Exemplary reaction conditions are found throughout the examples and in the literature.

In preferred embodiments, the glycan structures are remodeled so that the structure described in Formula 1 has specific determinates. The structure of the glycan can be chosen to enhance the biological activity of the peptide, give the peptide a new biological activity, remove the biological activity of peptide, or better approximate the glycosylation pattern of the native peptide, among others.

In the first preferred embodiment, the peptide N-linked glycans are remodeled to better approximate the glycosylation pattern of native human proteins. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:

X^3 and X^5 = |-GlcNAc-Gal-SA;

a and c = 1;

d = 0 or 1;

b, e and x = 0.

This embodiment is particularly advantageous for human peptides expressed in heterologous cellular expression systems. By remodeling the N-linked glycan structures to this configuration, the peptide can be made less immunogenic in a human patient, and/or more stable, among others.

In the second preferred embodiment, the peptide N-linked glycans are remodeled to have a bisecting GlcNAc residue on the tri-mannosyl core. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:

X^3 and X^5 are |-GlcNAc-Gal-SA;

a and c = 1;

X^4 is GlcNAc;

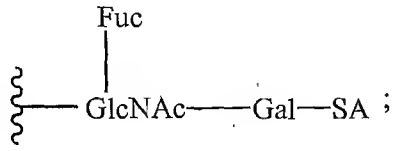
b=1;

d = 0 or 1;

e and x = 0.

This embodiment is particularly advantageous for recombinant antibody molecules expressed in heterologous cellular systems. When the antibody molecule includes a Fc-mediated cellular cytotoxicity, it is known that the presence of bisected oligosaccharides linked the Fe domain dramatically increased antibody-dependent cellular cytotoxicity.

In a third preferred embodiment, the peptide N-linked glycans are remodeled to have a sialylated Lewis X moiety. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:



X^3 and X^5 are ;

a, c, d = 1;

b, e and x= 0;

X^6= fucose.

This embodiment is particularly advantageous when the peptide which is being remodeling is intended to be targeted to selectin molecules and cells exhibiting the same.

In a fourth preferred embodiment, the peptide N-linked glycans are remodeled to have a conjugated moiety. The conjugated moiety may be a PEG molecule, another peptide, a small molecule such as a drug, among others. In this embodiment, the glycan structure described in Formula 1 is remodeled to have the following moieties:

X^3 and X^5 are |-GlcNAc-Gal-SA-R;

a and c = 1 or 2;

d = 0 or 1;

b, d, e and x = 0;

where R = conjugate group.

The conjugated moiety may be a PEG molecule, another peptide, a small molecule such as a drug, among others. This embodiment therefore is useful for conjugating the peptide to PEG molecules that will slow the clearance of the peptide from the patient's bloodstream, to peptides that will target both peptides to a specific tissue or cell, or to another peptide of complementary therapeutic use.

It will be clear to one of skill in the art that the invention is not limited to the preferred glycan molecules described above. The preferred embodiments are only a few of the many useful glycan moleeules that can be made by the remodeling method of the invention. Those skilled in the art will know how to design other useful glycans.

In the first exemplary embodiments, the peptide is expressed in a CHO (Chinese hamster ovarian cell line) according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from CHO cells, the N-linked glycans will have the structures depicted in top row of Figure 2, but also comprising a core fucose. While all of these structures may be present, by far the most common structures are the two at the right side. In the terms of Formula 1,

X^3 and X^5 are |-GlcNAc-Gal-(SA);

a and c = 1;

b, e and x = 0, and

d = 0 or 1.

Therefore, in one exemplary embodiment, the N-linked glycans of peptides expressed in CHO cells are remodeled to the preferred humanized glycan by contacting the peptides with a glycosyltransferase that is specific for a galactose acceptor molecule and a sialic acid donor molecule. This process is illustrated in Figure 2 and Example 17. In another exemplary embodiment, the N-linked glycans of a peptide expressed and secreted from CHO cells are remodeled to be the preferred PEGylated structures. The peptide is first contacted with a glycosidase specific for sialic acid to remove the terminal SA moiety, and then contacted with a glycosyltransferase specific for a galactose acceptor moiety and an sialic acid acceptor moiety, in the presence of PEG- sialic acid-nucleotide donor molecules. Optionally, the peptide may then be contacted with a glycosyltransferase specific for a galactose acceptor moiety and an sialic acid acceptor moiety, in the presence of sialic acid-nucleotide donor molecules to ensure complete the SA capping of all of the glycan molecules.

In other exemplary embodiments, the peptide is expressed in insect cells, such as the sf9 cell line, according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from sf9 cells, the N-linked glycans will often have the structures depicted in top row of Figure 6. In the terms of Formula 1:

X^3 and X^5 are |- GlcNAc;

a and c = 0 or 1;

b = 0;

X^6 is fucose,

d = 0, 1 or 2; and

e and x = 0.

The trimannose core is present in the vast majority of the N-linked glycans made by insect cells, and sometimes an antennary GlcNAc and/or fucose residue(s) are also present. Note that the glycan may have no core fucose, it may have a single core fucose having either linkage, or it may have a single core fucose with a perponderance of a single linkage. In one exemplary embodiment, the N-linked glycans of a peptide expressed and secreted from insect cells is remodeled to the preferred humanized glycan by first contacting the glycans with a glycosidase specific to fucose molecules, then contacting the glycans with a glycosyltransferases specific to the mannose acceptor molecule on each antennary of the trimannose core, a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules; then contacting the glycans with a glycosyltransferase specific to a GlcNAc acceptor molecule, a Gal donor molecule in the presence of nucleotide-Gal molecules; and then contacting the glycans with a glycosyltransferase specific to a galactose acceptor molecule, a sialic acid donor molecule in the presence of nucleotide-SA molecules. One of skill in the art will appreciate that the fucose molecules, if any, can be removed at any time during the procedure, and if the core fucose is of the same alpha 1,6 linkage as found in human glycans, it may be left intact. In another exemplary embodiment, the humanized glycan of the previous example is remodeled further to the sialylated Lewis X glycan by contacting the glycan further with a glycosyltransferase specific to a GlcNAc acceptor molecule, a fucose donor molecule in the presence of nucleotide-fucose molecules. This process is illustrated in Figure 11 and Example 39.

In yet other exemplary embodiments, the peptide is expressed in yeast, such as *Saccharomyces cerevisiae*, according to methods well known in the art. When a peptide with N-linked glycan consensus sites is expressed and secreted from *S. cerevisiae* cells, the N-linked glycans will have the structures depicted at the left in Figure 4. The N-linked glycans will always have the trimannosyl core, which will often be elaborated with mannose or related polysaccharides of up to 1000 residues. In the terms of Formula 1:

X^3 and X^5 = |-Man – Man – $(Man)_{0-1000}$;

a and c =1 or 2;

b, d, e and x = 0.

In one exemplary embodiment, the N-linked glycans of a peptide expressed and secreted from yeast cells are remodeled to the elemental trimannose core by first contacting the glycans with a glycosidase specific to α2 mannose molecules, then contacting the glycans with a glycosidase specific to α6 mannose molecules. This process is illustrated in Figure 4 and Example 38.

In another exemplary embodiment, the N-linked glycans are further remodeled to make a glycan suitable for an recombinant antibody with Fc-mediated cellular toxicity function by contacting the elemental trimannose core glycans with a glycosyltransferase specific to the mannose acceptor molecule on each antennary of the trimannose core and a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules. Then, the glycans are contacted with a glycosyltransferase specific to the acceptor mannose molecule in the middle of the trimannose core, a GlcNAc donor molecule in the presence of nucleotide-GlcNAc molecules and further contacting the glycans with a glycosyltransferase specific to a GlcNAc acceptor molecule, a Gal donor molecule in the presence of nucleotide-Gal molecules; and then optionally contacting the glycans with a glycosyltransferase specific to a galactose acceptor molecule and further optionally a sialic acid donor molecule in the presence of nucleotide-SA molecules. This process is illustrated in Figures 1, 2 and 3.

In another exemplary embodiment, the peptide is expressed in bacterial cells, in particular *E. coli* cells, according to methods well known in the art. When a peptide with N-linked glycans consensus sites is expressed in *E. coli* cells, the N-linked consensus sites will not be glycosylated. In an exemplary embodiment, a humanized glycan molecule is built out from the peptide backbone by contacting the peptides with a glycosyltransferase specific for a N-linked consensus site and a GlcNAc donor molecule in the presence of nucleotide-GlcNAc; and further sequentially contacting the growing glycans with glycosyltransferases specific for the acceptor and donor moieties in the present of the required donor moiety until the desired glycan structure is completed. When a peptide with N-linked glycans is expressed in a eukaryotic cells but without the proper leader sequences that direct the nascent peptide to the golgi apparatus, the mature peptide is likely not to be glycosylated. In this case

as well the peptide may be given N-linked glycosylation by building out from the peptide N-linked consensus site as aforementioned. When a protein is chemically modified with a sugar moiety, it can be built out as aforementioned.

These examples are meant to illustrate the invention, and not to limit it. One of skill in the art will appreciate that the steps taken in each example may in some circumstances be able to be performed in a different order to get the same result. One of skill in the art will also understand that a different set of steps may also produce the same resulting glycan. The preferred remodeled glycan is by no means specific to the expression system that the peptide is expressed in. The remodeled glycans are only illustrative and one of skill in the art will know how to take the principles from these examples and apply them to peptides produced in different expression systems to make glycans not specifically described herein.

B. Method to remodel O-linked glycans

O-glycosylation is characterized by the attachment of a variety of monosaccharides in an O-glycosidic linkage to hydroxy amino acids. O-glycosylation is a widespread post-translational modification in the animal and plant kingdoms. The structural complexity of glycans O-linked to proteins vastly exceeds that of N-linked glycans. Serine or threonine residues of a newly translated peptide become modified by virtue of a peptidyl GalNAc transferase in the cis to trans compartments of the Golgi. The site of O-glycosylation is determined not only by the sequence specificity of the glycosyltransferase, but also epigenetic regulation mediated by competition between different substrate sites and competition with other glycosyltransferases responsible for forming the glycan.

The O-linked glycan has been arbitrarily defined as having three regions: the core, the backbone region and the peripheral region. The "core" region of an O-linked glycan is the inner most two or three sugars of the glycan chain proximal to the peptide. The backbone region mainly contributes to the length of the glycan chain formed by uniform elongation. The peripheral region exhibits a high degree of structural complexity. The structural complexity of the O-linked glycans begins with the core structure. In most cases, the first sugar residue added at the O-linked glycan consensus site is GalNAc; however the sugar may also be GlcNAc, glucose, mannose, galactose or fucose, among others. Figure 12 is a

diagram of some of the known O-linked glycan core structures and the enzymes responsible for their in vivo synthesis.

In mammalian cells, at least eight different O-linked core structures are found, all based on a core-α-GalNAc residue. The four core structures depicted in Figure 13 are the most common. Core 1 and core 2 are the most abundant structures in mammalian cells, and core 3 and core 4 are found in more restricted, organ-characteristic expression systems. O-linked glycans are reviewed in Montreuil, Structure and Synthesis of Glycopeptides, In Polysaccharides in Medicinal Applications, pp. 273-327, 1996, Eds. Severian Damitriu, Marcel Dekker, NY, and in Schachter and Brockhausen, The Biosynthesis of Branched O-Linked Glycans, 1989, Society for Experimental Biology, pp. 1-26 (Great Britain).

It will be apparent from the present disclosure that the glycan structure of O-glycosylated peptides can be remodeled using similar techniques to those described for N-linked glycans. O-glycans differ from N-glycans in that they are linked to a serine or threonine residue rather than an asparagine residue. As described herein with respect to N-glycan remodeling, hydrolytic enzymes can be used to cleave unwanted sugar moieties in an O-linked glycan and additional desired sugars can then be added thereto, to build a customized O-glycan structure on the peptide (See Figures 12 and 13).

The initial step in O-glycosylation in mammalian cells is the attachment of N-acetylgalactosamine (GalNAc) using any of a family of at least eleven known α-N-acetylgalactosaminyltransferases, each of which has a restricted acceptor peptide specificity. Generally, the acceptor peptide recognized by each enzyme constitutes a sequence of at least ten amino acids. Peptides that contain the amino acid sequence recognized by one particular GalNAc-transferase become O-glycosylated at the acceptor site if they are expressed in a cell expressing the enzyme and if they are appropriately localized to the Golgi apparatus where UDP-GalNAc is also present.

However, in the case of recombinant proteins, the initial attachment of the GalNAc may not take place. The α-N-acetylgalactosaminyltransferase enzyme native to the expressing cell may have a consensus sequence specificity which differs from that of the recombinant peptide being expressed.

The desired recombinant peptide may be expressed in a bacterial cell, such as *E. coli,* that does not synthesize glycan chains. In these cases, it is advantageous to add the initial

GalNAc moiety *in vitro*. The GalNAc moiety can be introduced *in vitro* onto the peptide once the recombinant peptide has been recovered in a soluble form, by contacting the peptide with the appropriate GalNAc transferase in the presence of UDP-GalNAc.

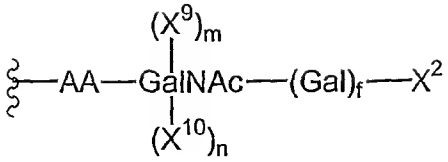
In one embodiment, an additional sequence of amino acids that constitute an effective acceptor for transfer of an O-linked sugar may be present. Such an amino acid sequenee is encoded by a DNA sequence fused in frame to the coding sequence of the peptide, or alternatively, may be introduced by chemical means. The peptide may be otherwise lacking glycan chains. Alternately, the peptide may have N- and/or O-linked glycan chains but require an additional glycosylation site, for example, when an additional glycan substituent is desired.

In an exemplary embodiment, the amino acid sequence PTTTK-COOH, which is the natural GalNAc acceptor sequence in the human mucin MUC-1, is added as a fusion tag. The fusion protein is then expressed in *E. coli* and purified. The peptide is then contacted with recombinant human GalNAc-transferases T3 or T6 in the presence of UDP-GalNAc to transfer a GalNAc residue onto the peptide *in vitro*.

This glycan chain on the peptide may then be further elongated using the methods described in reference to the N-linked or O-linked glycans herein. Alternatively, the GalNAc transferase reaction can be carried out in the presence of UDP-GalNAc to which PEG is covalently substituted in the O-3, 4, or 6 positions or the N-2 position. Glycoconjugation is described in detail elswhere herein. Any antigenicity introduced into the peptide by the new peptide sequence can be conveniently masked by PEGylation of the associated glycan. The acceptor site fusion technique can be used to introduce not only a PEG moiety, but to introduce other glycan and non-glycan moieties, including, but not limited to, toxins, anti-infectives, cytotoxic agents, chelators for radionucleotides, and glycans with other functionalities, such as tissue targeting.

Exemplary Embodiments

The remodeling of O-linked glycosylation is best illustrated with reference to Formula 2:



Formula 2 describes a glycan structure comprising a GalNAc which is covalently linked preferably to a serine or threonine residue on a peptide backbone. While this structure is used to illustrate the most common forms of O-linked glycans, it should not be construed to limit the invention solely to these O-linked glycans. Other forms of O-linked glycans are illustrated in Figure 12. Preferred expression systems useful in the present invention express and secrete exogenous peptides having O-linked glycans comprising the GalNAc residue. Using the remodeling methods of the invention, the glycan structures on these peptides can be conveniently remodeled to generate any desired glycan structure. One of skill in the art will appreciate that O-linked glycans can be remodeled using the same principles, enzymes and reaction conditions as those available in the art once armed with the present disclosure. Exemplary reaction conditions are found throughout the Examples.

In preferred embodiments, the glycan structures are remodeled so that the structure described in Formula 2 has specific moieties. The structure of the glycan may be chosen to enhance the biological activity of the peptide, confer upon the peptide a new biological activity, remove or alter a biological activity of peptide, or better approximate the glycosylation pattern of the native peptide, among others.

In the first preferred embodiment, the peptide O-linked glycans are remodeled to better approximate the glycosylation pattern of native human proteins. In this embodiment, the glycan structure described in Formula 2 is remodeled to have the following moieties:

X^2 is |-SA; or |-SA-SA;

f and n = 0 or 1;

X^{10} is SA;

m = 0.

This embodiment is particularly advantageous for human peptides expressed in heterologous cellular expression systems. By remodeling the O-linked glycan structures to have this configuration, the peptide can be rendered less immunogenic in a human patient and/or more stable.